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EFFECTS OF COPPER ON THE LOCOMOTOR ORIENTATION OF FISH

H. Kleerekoper

Texas A and M University

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Effects of Copper on the Locomotor Orientation of Fish

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~~*Locomotion in fish~~ Fish behavior,
~~*Orientation in fish~~ Water pollution effects, *Effect of gradient, (streams) ~~slope on orientation~~
~~*Avoidance behavior in fish~~ ~~*Temperature and orientation~~

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White sucker, Carassius auratus, Avoidance behavior (fish)
~~channel catfish~~ ~~Catostomus commersoni commersoni~~
green sunfish, Micropterus salmoides,
~~largemouth bass~~ ~~Ictalurus punctatus~~,

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by

H. Kleerekoper
Texas A&M University
Department of Biology
College Station, Texas 77843

Project #R800995 (18050 DWQ)

Project Officer

Dr. James M. McKim
National Water Quality Laboratory
Environmental Protection Agency
6201 Congdon Boulevard
Duluth, Minnesota 55804

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ABSTRACT

The effects of copper ions at subacute concentrations on the locomotor orientation of goldfish (Carassius auratus), channel catfish (Ictalurus punctatus), largemouth bass (Micropterus salmoides), white sucker (Catostomus commersoni commersoni) and green sunfish (Lepomis cyannelus) were investigated in detail.

In regions of water containing 11-17 µg/l Cu^{++} (as $CuCl_2$) in a shallow gradient goldfish oriented toward the copper source ("attraction"). This response is reduced in a somewhat steeper gradient. In steep gradients significant but no absolute avoidance behavior occurred. Whether the response will be "avoidance" or "attraction" seems to depend on the slope of the gradient to which the fish is exposed. Even in steep gradients, the "avoidance" behavior is reversed to "attraction" when the copper ions interact with a temperature slightly higher (.4C) than that of the surrounding copper free water. The interaction creates a new stimulus configuration which is different from those formed by the two variables separately.

The orientation of the largemouth bass is not affected by copper ions at the concentrations tested. Channel catfish are weakly attracted by the copper-containing water and green sunfish significantly increase time spent there. Suckers significantly but not absolutely "avoid" such water through changes in turning behavior.

The report was submitted in fulfillment of Project Number R800995 (18050 DWQ) under the sponsorship of the Water Quality Office, Environmental Protection Agency.

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SECTION I

CONCLUSIONS

In some species of fish the locomotor response to subacute concentrations of copper ions results in changes in orientation. The net result of these changes may be "attraction" or "avoidance", orientation towards copper-containing zones of water. The evidence indicates that the direction of the orientation is relatively independent of the maximum concentration of the metal but is determined particularly by the gradient of copper ions as the fish pass from laboratory water to copper ion containing water. A shallow gradient leading to the zones of maximum copper concentration elicits a clear "attraction", a medium steep gradient decreases that response somewhat, and a steep gradient results in significant "avoidance". However, in the latter condition, highly significant "attraction" is again obtained when a temperature rise of .4C is associated with the copper ion. Copper and temperature interact to elicit orientation response not produced by either copper alone or temperature alone. Responses of this type can only be assessed through detailed monitoring of locomotor characteristics and careful data analysis.

SECTION II

RECOMMENDATIONS

It is recommended that the interaction of environmental variables be emphasized in future studies. It is most unlikely that in natural conditions variations in a single variable occur without simultaneous variations of one or more other variables. Thus, new stimulus configurations arise, the animal's response to which may be entirely different from the response to variations of the individual components of the configuration.

The "typical" experimental procedure: "all conditions constant except the variable under study" ignores the possible interaction of variables as they affect the animal's response.

The heat-copper interaction reported here provides clear evidence for the need to analyze the effects of interaction of commonly occurring environmental variables on the locomotor behavior of fish.

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SECTION III

INTRODUCTION

The very extensive literature on the effects of heavy metal ions on fish deals almost exclusively with various aspects of the acute effects of exposure to these substances. Only recently has attention been directed to chronic effects of subacute concentrations of salts of various heavy metals. Most of the available information in this area refers to effects of exposure to heavy metals on the physiology and viability of fish. Very scarce indeed is quantitative information on the behavioral toxicology of these substances as it affects locomotor behavior, particularly locomotor orientation. This gap in knowledge is especially serious in the assessment of the potential harm to fishes of heavy metals in the environment.

The potential danger for fish of any toxic substance in the environment is greatly affected by the orientation behavior which the substance elicits in the animals. A naive fish, that is a fish not previously exposed to the substance under study, may spontaneously respond to the perception of the toxic by orienting away from the source ("avoidance" behavior), by approaching it ("attraction"), or by "ignoring" it insofar as orientation is concerned. Obviously, a polluting substance responded to by effective "avoidance" orientation has a far lesser harmful potential than one that elicits "attraction" or is responded to indifferently.

The research here reported addresses itself to this and some other problems.

The theoretical and experimental approaches were designed to acquire quantitative information of the highest attainable reliability on the locomotor orientation in various experimental conditions and to treat this information with the best available statistical techniques so that the results might not only inspire confidence but constitute bases for generalization applicable to field conditions. In pursueing this aim two major points had to be considered. 1. locomotor behavior is highly variable over time, requiring the monitoring of the behavior over prolonged periods for all experimental conditions. 2. oriented behavior only exceptionally displays an obvious directionality, recognizable by more or less casual, descriptive techniques or by direct observation over short periods. Some of the results reported here make this clear. The orientation of the locomotor behavior of fish is affected only subtly by changes in the environment. Changes in frequency and sizes of turns, length of steps between turns, time of sojourn in environmentally differents areas, velocity, and other parameters constitute most often the only behavioral responses to environmental change. The totality of these responses over time

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determine the final change in locomotor orientation, that is, whether "avoidance" or "attraction" will occur. The experimental and analytical complexities resulting from this behavior as it relates to the problems with which we are concerned here are formidable. Consequently, much preliminary experimentation, testing of statistical techniques on the preliminary data acquired, designing and "debugging" of computer programs constitute the massive background, not readily discernable by the casual reader, of the research reported in the following sections which represents 106 experiments with five species of fish.

SECTION IV

EXPERIMENTAL RATIONALE

The experimental design of the research here reported aimed at obtaining accurate quantitative descriptions of the locomotor behavior of the experimental fish prior to and during the exposure to subacute concentrations of copper ions in a free choice, "open field" situation. The data treatment was designed to assess the differences in the behavior as the fish moved from laboratory water to copper-containing water. Locomotor behavior can be quantitatively described when the following parameters are known: 1. magnitude of turns; 2. length of straight paths or steps between turns; 3. absolute orientation of the fish at each displacement; 4. velocity.

Variability in time of these parameters requires long term observations which can only be obtained through automatic monitoring and data acquisition. Two monitoring tanks were used, each yielding information of different but complementary nature and requiring specific treatment of the data obtained from each monitor.

The monitoring systems used in this research consist of: A. data acquisition and processing equipment, and B. a number of peripheral mechanisms to control and monitor the environmental parameters to be investigated. Part A comprises: 1. two experimental tanks which monitor the locomotor activity of a fish, and 2. computation and data processing systems including a number of computer programs.

Monitor Tank #1: It measures 5.00 x 5.00 x .5m and holds, embedded in its floor, a square matrix of 1936 photoconductive cells on 10 cm centers, their light sensitive faces directed upwards (Fig. 1). The cells are activated by collimated light which, from overhead, illuminates, at 2500 K, the whole surface of the tank uniformly, and at low intensity. Collimation is obtained through Fresnel lenses which cover a flat black aluminum honeycomb, 5 cm deep, suspended horizontally over the whole area of the tank (Fig. 2). The photoconductive cells were selected for peak response in the far red so that the for the fish eye visible part of the spectrum may be filtered out without affecting the operation of the sensors. Interception of light by the presence of the fish over a photocell increases the resistance of the cell, an event which affects an electronic logic circuit, formed by the x and y axes of the cell matrix. This circuit interfaces with the data acquisition system outlined below.

Figure 1

Monitor Tank # 1

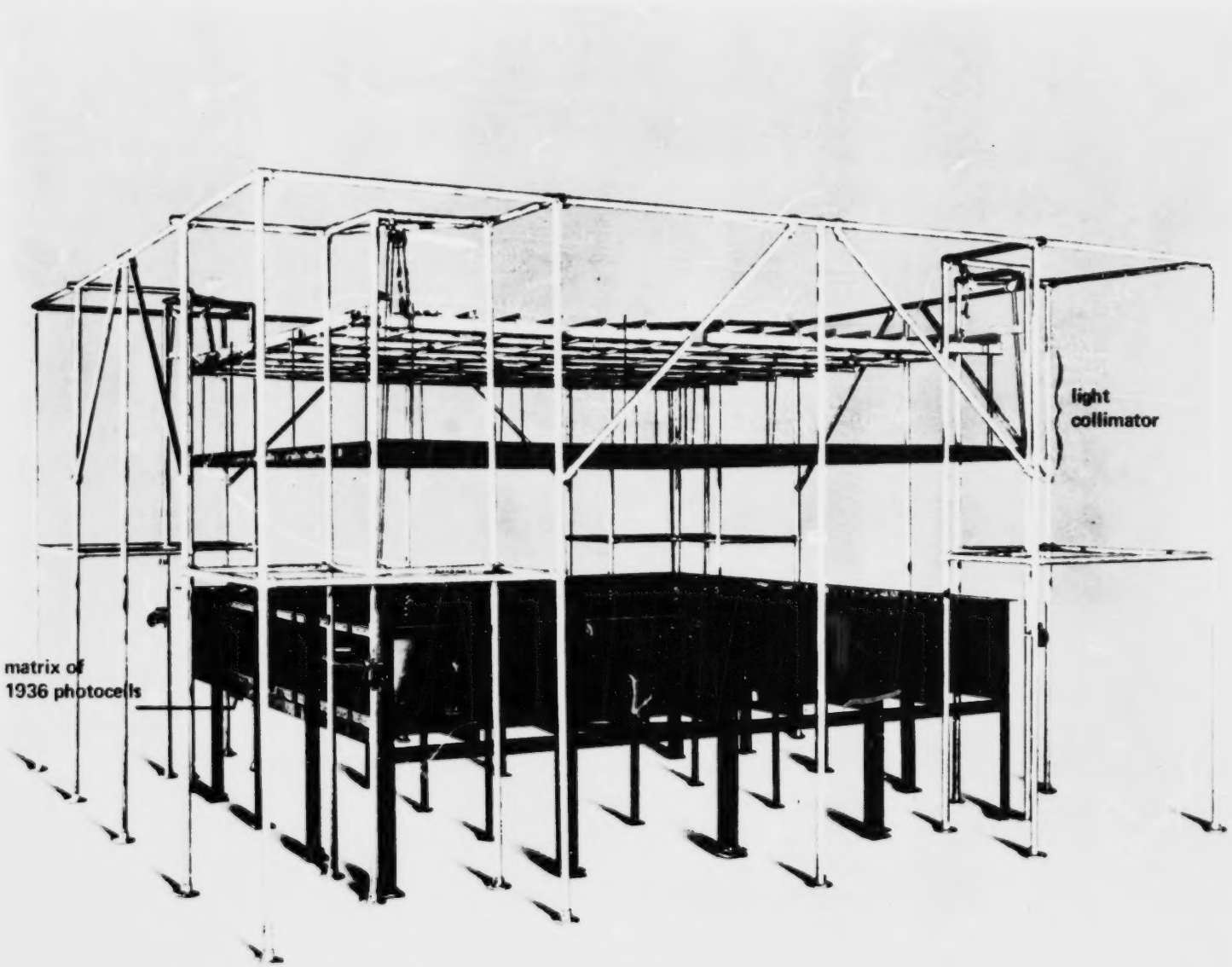
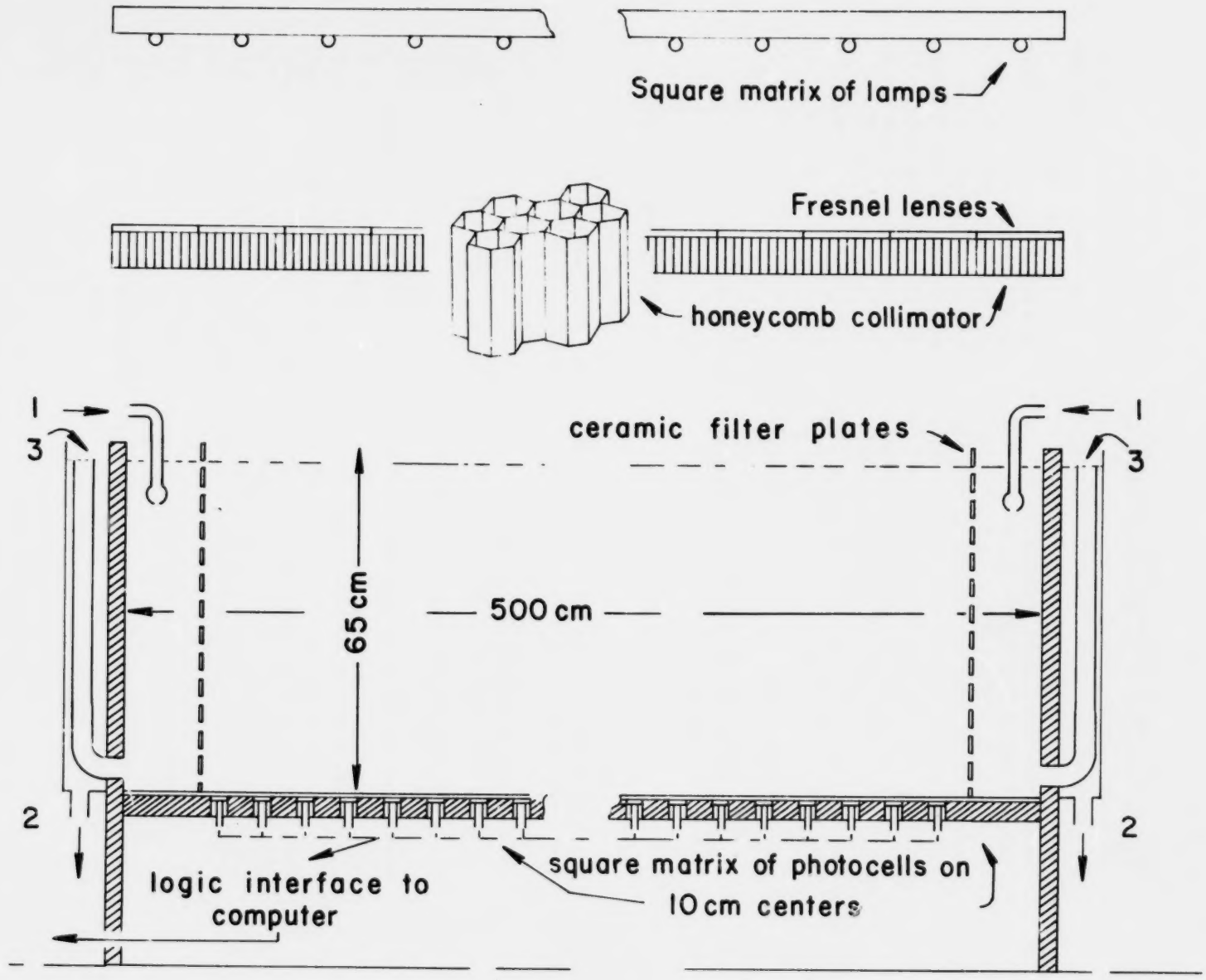

light
collimator
matrix of
1936 photocells

Figure 2

Schematic Cross Section of Monitor Tank #1


Square matrix of lamps
Fresnel lenses
honeycomb collimator
1
3
ceramic filter plates
65 cm
500 cm
2
logic interface to computer
square matrix of photocells on 10 cm centers

The tank is isolated from all control and processing equipment, and is located in a room which, like the tank itself, is painted a flat black throughout.

Data Acquisition and Processing: A small computer is interfaced on-line with the cell matrix of the monitor and is programmed to locate the position in the matrix of the photocell affected by the presence of the fish. Simultaneously, a real time clock in the system determines the time elapsed between the event in question and the immediately preceding event. These three coordinates, x, y, and time, form the basis for a series of computations by the computer which determine direction and size of angles of turn, distance between successive photocells shaded by the fish, velocity of locomotion, absolute direction of the movements in the tank, length of step between successive turns and "handedness" of the animals. Angle resolution is better than 4° for 30 cm long goldfish. These basic computations could be printed out on a teletypewriter but were more commonly recorded on magnetic tape. In addition, the displacements of the fish could be displayed on a cathode ray tube (Fig. 3).

The magnetic tape record was used, as needed,

1. To obtain from a plotter a graphic record of the locomotor pattern during all or part of an experiment;

2. to feed back data to the computer for additional computations;

3. to feed back to an IBM 360 computer, available on campus, for the execution of a variety of programs which were designed to analyze the data collected from a variety of viewpoints. (see below).

Peripheral Equipment and Mechanisms to Control and Monitor Environmental Conditions Related to the Problems of this Research

1. Water supply and flow control.

Water is admitted to the tank through any one of four peripheral channels, located along the four walls of the tank, which are in open communication with the tank through porous plates (Fig. 2). It then enters the main body of the tank through the plates along one side and leaves through the plates along the opposite side of the tank. This arrangement brings about an almost perfect laminar flow across the tank. The direction of flow and its rate can be regulated through remote control from an adjacent room.

2. Temperature control.

The temperature of the supply water was controlled at 21 C $\pm$ 1C.

Treatment of Data from Monitor #1. The data obtained from the monitor, recorded on magnetic tape, supplied basic information on the locomotor behavior prior to and after the experimental change and provided a plotter-produced tracing of the locomotor pattern. To this effect, all or some of the following computer programs, were applied.

1. Event by event printout including x, y, time, distance, velocity, angle in tank, change in angle.

2. Hour by hour breakdown including turns per hour, percent right, percent left, distance per hour, percent without turns, average length of straight line, average velocity.

3. Frequency distribution of the changes in direction for a range of 0 - 180° in 10° classes.

Frequency distribution of the velocities for a range of 0 - 95 cm/sec. in 5 cm/sec. classes.

4. Frequency distribution of the distances travelled between events for a range of 0 - 120 cm in 10 cm classes.

5. F statistic for analysis of variance of group sizes from 2 to 50 for the first N events.

6. Revisitation of cells including a printout of all the cells in the tank and the number of times each was fired plus a listing of the number of cells fired one time, two times, three times, etc.

7. Frequency distribution of the changes in direction (range 0 - 180°) in 10° classes in one hour periods. These distributions could be cumulated hour by hour.

Frequency distribution of velocities (range 0 - 95 cm/sec for 5 cm/sec classes) in one hour periods. These distributions could be cumulated hour by hour.

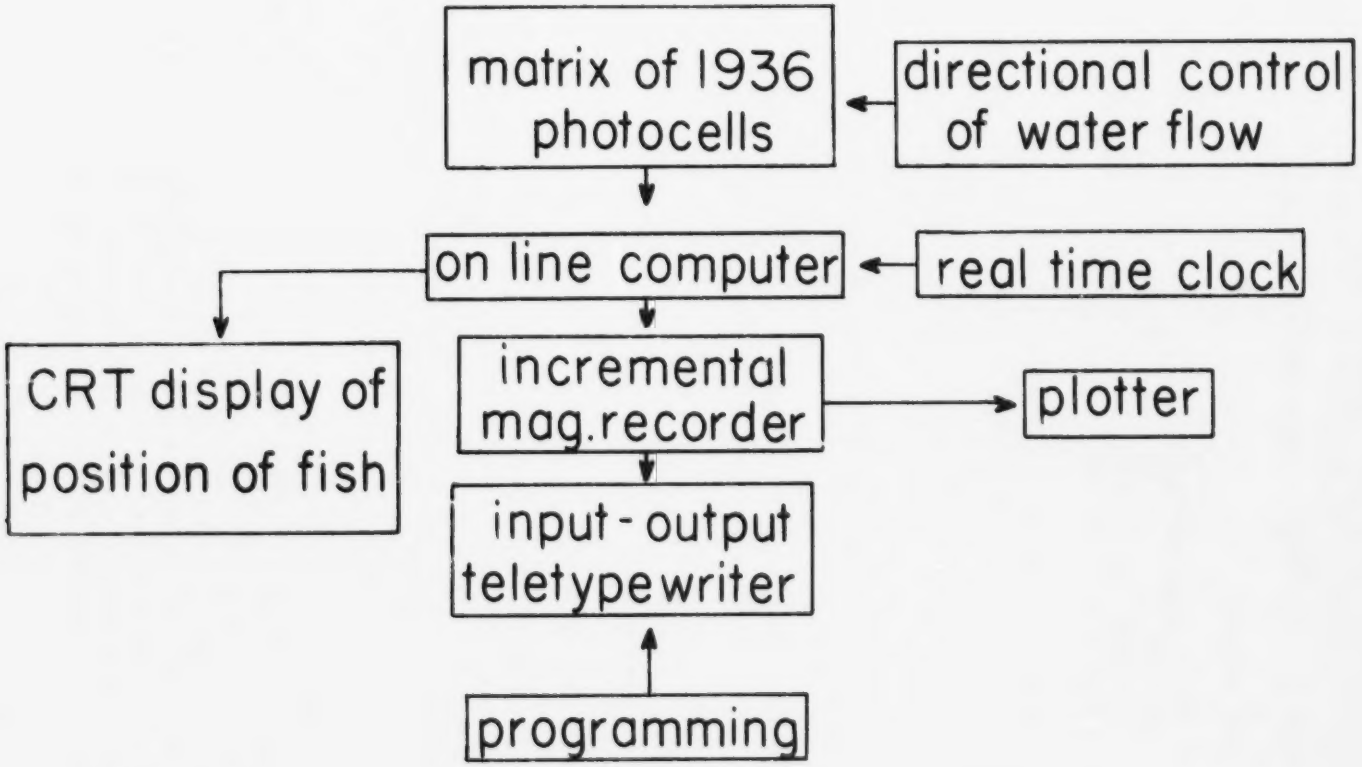
8. Contingency table on the relation of consecutive angles of turn.

9. For classes of 1000 events: the number of changes in direction, the average change in direction without regard to sign, the average length of a straight path and the average velocity.

10. Distribution of distances travelled with respect to orientation. Orientation is expressed as 10° classes from 0 - 360° (actually 9.75° classes with 359.75° to 360° included with 0° class).

Figure 3

Diagram of Interfacing Between the Monitor, Computer, and Peripheral Data-processing Components


matrix of 1936 photocells
directional control of water flow
on line computer
real time clock
CRT display of position of fish
incremental mag.recorder
plotter
input - output teletypewriter
programming

11. Distribution of distances travelled with respect to orientation. Orientation is expressed as 10° classes from 0 - 360° (actually 9.75° classes with 359.75° to 360° included with 0° class).

12. Correlation coefficient of incoming angle to outgoing angle in 10 cm distance classes for a range of 0 - 500 cm.

13. Orthogonal polynomial surface fit (see below).

"Block-time" listing

Any one of these programs, where applicable, could be restricted to data from specific parts of the tank so that the spatial effects of variable gradients, currents as well as that of artefacts (the walls and corners of the tank) could be analyzed.

Special attention is called to program 13 which allowed us to test the statistical significance of changes in any one of the locomotor parameters mentioned with a very high degree of sensitivity and to represent the change spatially as a surface fit representing the total area of the tank.

In view of the important interpretative role this test played in the research, an outline of the procedure follows.

The square tank was subdivided into a 7 x 7 area and for each subdivision the following (X) characteristics are derived:

1) frequency

2) straight path length

3) other characteristics

The three-fold statistical objective was 1) to fit an empirical polynomial model for each characteristic, 2) to test the equality of such surfaces over different treatments, and 3) to interpret the differences in surfaces provided they are statistically significant. The above analysis was performed for each of the (X) characteristics individually. Each phase is discussed separately below.

Polynomial Model: The data for a particular characteristic, call it Z, consist of individual responses, Z_{ij}, for each of the seven equidistant X_i coordinates of the tank and for each of the seven equidistant Y_j coordinates. This two-dimensional layout is statistically benign in permitting the use of two-dimensional orthogonal polynomials. Such polynomials are not only computationally efficient and accurate but also allow the order of the necessary fit equation to be readily determined. Preliminary analyses of the various characteristics suggested in most cases both the statistical necessity

as well as sufficiency of a fifth order model. Hence the following analysis assumes the fifth order equation; any other order would modify subsequent discussion only slightly.

The resultant equation may be written as:

$$Z_{ij} = B_{oo} + B_{10} X_i + B_{01} Y_j + B_{20} X_j^2 + B_{11} X_i X_j$$

$$+ B_{02} Y_j^2 + \ldots + B_{50} X_i^5 + \ldots.$$

$$+ B_{14} X_i Y_j^4 + B_{05} Y_j^5 + e_{ij}$$

for $i = 1, \ldots, 7$ and $j = 1, \ldots, 7$

where

Z_{ij} denotes the Z response of the i, j^{th} cell

X_i denotes the i^{th} position on the X axis

Y_j denotes the j^{th} position on the Y axis and

e_{ij} denotes the error term of the i, j^{th} cell.

The variances of the observations are assumed equal and the covariances slight, hence the ordinary least squares method is used to fit the above 21 coefficients to the 49 data.

Surface Equality. The next phase of the statistical analysis tested the null hypothesis of whether the three surfaces were equal for different treatments. The standard procedure for linear regression models (see e.g. Ostle (1963) p. 201) may be generalized to multiple regression by 1) fitting the above equation to the three sets of 49 responses simultaneously and then 2) comparing that residual sum of squares to the total of the individual sums of squares. An F test was then performed which in this case has 42 and 84 degrees of freedom.

Region of Difference. The third phase was conducted upon the rejection of the equality of surfaces hypothesis above the attempts to identify the source of the significant differences. The treatment was applied in such a manner that only certain of the 49 total cells were affected. Based on this and prior knowledge, the tank was divided into several regions of interest according to the concentration of treatment and tank location. The third phase investigates whether the pattern of residuals changed from region to region. An affirmative answer to the above question suggested that such changes caused the difference in polynomial fits.

Since fish activity varied a great deal from cell to cell, the cells of a specific region were considered individually to overcome the

cell-to-cell variability by pairing. For a particular cell, the response of the water treatment was compared with the copper treatment. Two paired non-parametric tests, the sign test and the signed rank test, tested the hypothesis of a difference in response for that given region. Also the homogeniety of the response over the different regions was tested by the chi-square statistic.

Monitor # 2: This equipment is illustrated in figure 4. Cylindrical tank (9) is made of acrylic and has double walls, 50 cm high, which form a peripheral channel, 7.5 cm wide (17). The central remainder of the tank has a diameter of 200 cm. Sixteen hollow dividers, 50 cm long; 5 cm wide, 35 cm high, extend radially from the inner wall toward the center to create 16 peripheral compartments of equal dimensions and an open central area. The dividers contain light sources and photocell arrays with peak response at 700 nm and form photoelectric gates which are activated by the passage of a fish. The peripheral channel (17), receives fresh water of which the ion content can be controlled and varied or synthetic seawater. Either medium is treated through rapid and diatomaceous earth filters. At the peripheral center of each compartment water enters the equal rate from the channel (17) through siphons and leaves centrally through pipe 18. Entries and exits of compartments by the fish are monitored as to compartment number, solar time of entry and time of exit, by the electronic interface and recorded through a paper punch for computer processing (Kleerekoper, 1967, 1969, Kleerekoper et al., in press).

The tank rests on a neoprene vibration-damping pad (11) on a platform (12). On the ceiling of the experimental room a bank of 36 fluorescent lamps (1) is mounted whose light passes through a diffuser (2) of white translucent plexiglas and a cloth of white cotton (3). Curtain 8 provides for a lightproof connection between tank and "sky". No extraneous light enters the experimental room.

To produce temperature differentials between compartments heating pads were used. Each pad is a nearly flat, black, silicone rubber mat in which resistance wire is embedded the electrical power to which was controlled.

Flow rate in each of 16 compartments was regulated individually.

Statistical Procedures, Monitor 2: Temperature and flow rates were arranged so as to obtain a variation of conditions in the 16 compartments; the percent distributions of the frequency of entry and time spent in each compartment were analyzed in a fractional factorial design.

Variability between fish was eliminated by blocking and bias due to intangible physical factors was minimized through randomized assignment of experiment conditions to the 16 compartments. Variability

due to conditions was partitioned into contrasts and selected treatment comparisons to isolate the effects of each.

Water Supply: Both montitors were supplied with continuously flowing tap water filtered through activated carbon. A representative chemical analysis of this water is shown in Table 1.

Introduction of Chemicals: Glass syringes, actuated by a multichannel infusion-withdrawal pump mechanism of continuously variable speed under servo control, withdrew a concentrated solution of either sodium chloride or copper chloride from a supply vessel and delivered the solution at the required rate through silastic tubing and stainless steel fittings into the monitors.

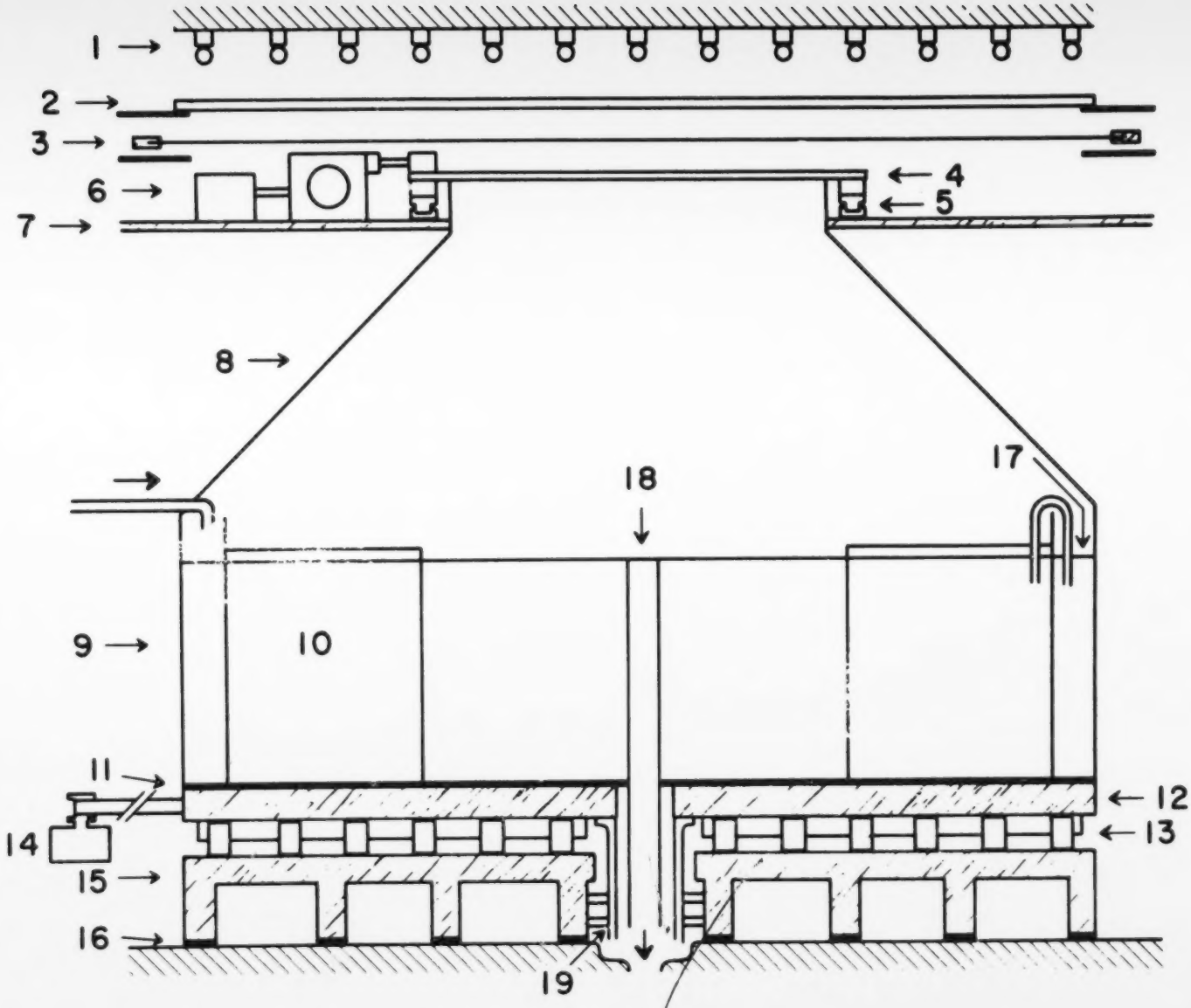
For monitor #1 the solutions were introduced through the perforated water pipe supplying 1/3 of the length of one channel (Fig. 5). Individual compartments of monitor #2 received the chemical solutions through side arms of the glass water supply tubes.

Measurements of Ion Distribution in the Tank: Since a major aspect of the research concerned the effects of copper ion distribution on the orientation of locomotion, accurate information was required on the distribution patterns of the ion in the tank. This was measured as follows. Thermistors and conductivity cells of the flow-through type were mounted horizontally in pairs (one thermistor and one cell), in a rectangular matrix on 10 x 20 cm centers, on an open supporting rack. The dimensions of the latter were those of the vertical cross section of the water in the tank and allowed for a grid of 5 rows (10 cm interval) and 23 columns (20 cm interval) of sensor pairs. These were connected through an interface to the computer, which was programmed to record electrolytic conductivity and temperature, together with the positions of the sensors, on magnetic tape, and/or to print out these results on the teletypewriter. The temperature data allowed for correction of the conductivity readings of the individual cells when slight temperature differences occurred. The data required for the construction of the profiles of ion distributions were obtained by placing the grid of sensors vertically in the tank and recording the conductivity and temperature values registered by the 115 pairs of sensors, an operation which required less than 5 minutes. Vertical conductivity profiles were recorded at various distances from the water inlet. At the start of each recording, sodium chloride solution was introduced into the water entering section B (Fig. 5) of the water supply channel at a constant rate. Recordings were repeated at 5 minute intervals.

The Experimental Fish: Prior to the experiments, the fish were kept in the laboratory in large holding tanks in water at 18-20°C continuously circulating through filters of activated carbon. The fish were fed with "trout chow" while in the holding tanks but were not fed in the monitor tanks, in which they remained only during the recording periods.

Figure 4

Schematic Cross Section of Monitor Tank # 2


1
2
3
4
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6
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9
10
11
12
13
14
15
16
17
18
19

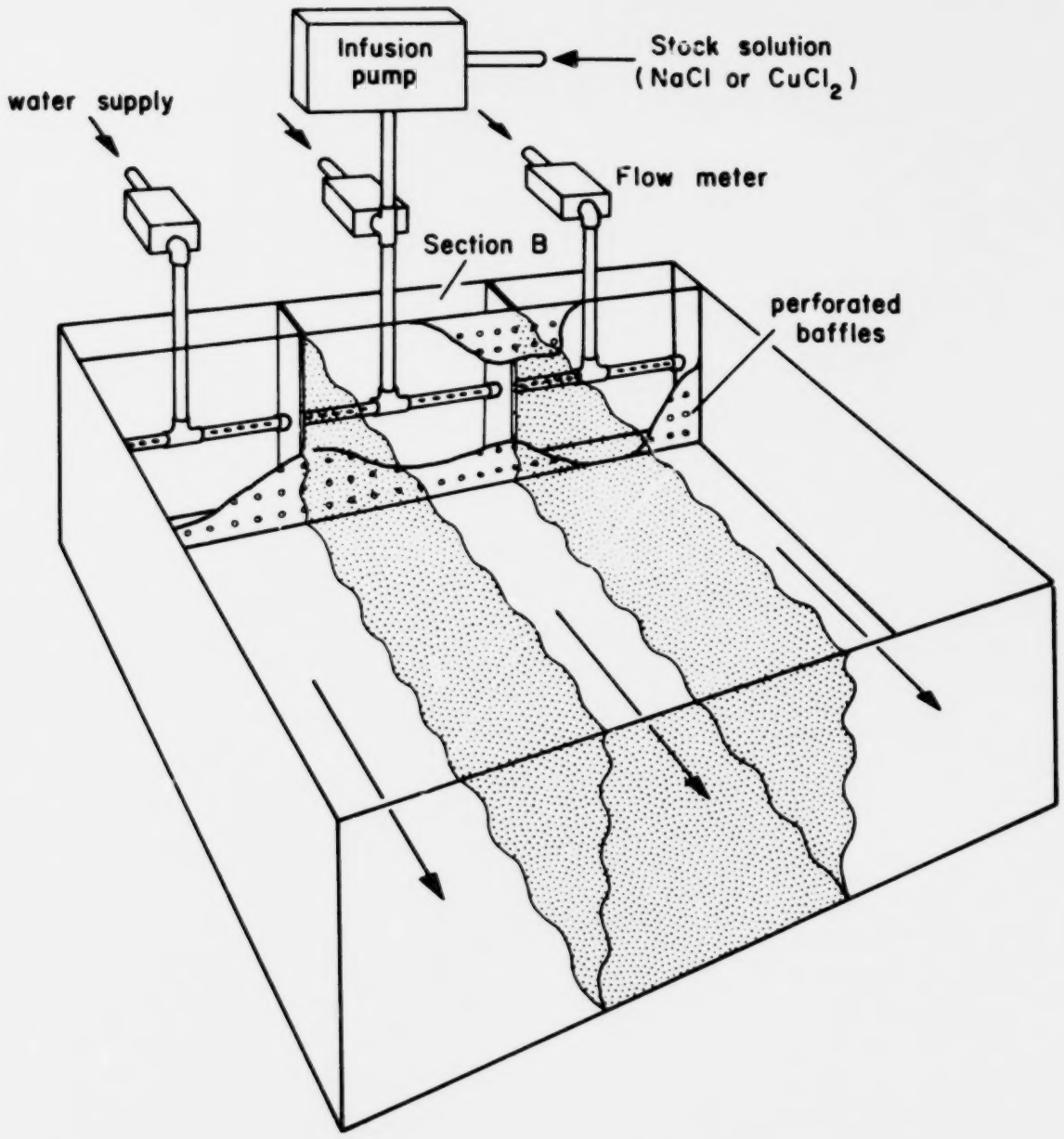
The species used and the origin of the fish will be mentioned separately for each series of experiments.

TABLE 1. Chemical characteristics (mg/liter except as indicated) of the laboratory water.

Carbonates ($-CO_3$)	10
Bicarbonates ($-HCO_3$)	152
Chlorides	47.5
Sulphates ($-SO_4$)	10.7
Silica (SiO_2)	18.5
Ca^{++}	1.8
Mg^{++}	0.2
Na^{+}	99.4
Hardness ($CaCO_3$)	5.4
Cu^{++}	0.006
Zn^{++}	0.002
Conductivity µmho	162
pH	8.4

Figure 5

Schematic View of the Water Supply and Copper Infusion System of Monitor # 1


Infusion pump
Stock solution (NaCl or $CuCl_2$)
water supply
Flow meter
Section B
perforated baffles

SECTION V

THE ORIENTATION OF GOLDFISH (CARASSIUS AURATUS) IN RESPONSE TO A SHALLOW GRADIENT OF A SUBLETHAL CONCENTRATION OF COPPER IN AN OPEN FIELD

Knowledge of the locomotor responses of fish to concentrations of heavy metals was based mainly on observation of animals exposed to steep gradients of these substances produced experimentally in narrow troughs. The aim of this part of the study was to analyze the locomotor response to shallow gradients of sublethal concentrations of copper ions (as $CuCl_2$) in an open field situation, in conditions of uniform, slow flow (1.8 cm/s), by 30 cm long goldfish.

For this part of the study monitor #1 was used.

Glass syringes, actuated by a multi-channel infusion-withdrawal pump mechanism of continuously variable speed under servo control withdrew a concentrated solution of either sodium chloride or copper chloride from a supply vessel and delivered the solution at the required rate through silastic tubing and stainless steel fittings into the water pipe (PVC) supplying 1/3 of the length of one channel (Figure 5).

The progression of sodium or copper chloride ions introduced into section B of the water supply channel (Figure 5) is summarized in figure 6. Conductivity values were recorded in 5 minute intervals in the planes indicated in the figure while the water traversed the tank at a rate of 1.8 cm/s. Figure 6 represents the conductivity pattern at the end of one and two hours following the introduction of the chloride with a resolution of six micromhos. The lighter shade of grey corresponds to the background conductivity of the supply water prior to the introduction of the chloride, successively darker shades representing increases in steps of six micromhos. The condition towards the end of the third hour (not shown in the figure) indicated that only small pockets of water remained unaffected by the chloride. During the first two hours, however, the chloride-enriched water moved across the tank as a mass whose boundaries are indicated in the profiles shown in figure 6 and which was flanked by "clean" water.

The information thus acquired allowed for the distinction and comparison of the characteristics of locomotion within (Region B) and outside (Region A) the water masses "polluted" by copper ions. Furthermore, this information established that such comparison should be most effective for data collected during a period not exceeding two hours following the onset of the "pollution", that is, when polluted and non-polluted regions in the tank were still well defined. The effects of the movements of the fish on the patterns of the boundaries between the two regions are unknown. However, the unidirectional, quasi laminar flow should tend to continuously correct local disturbances produced by fish.

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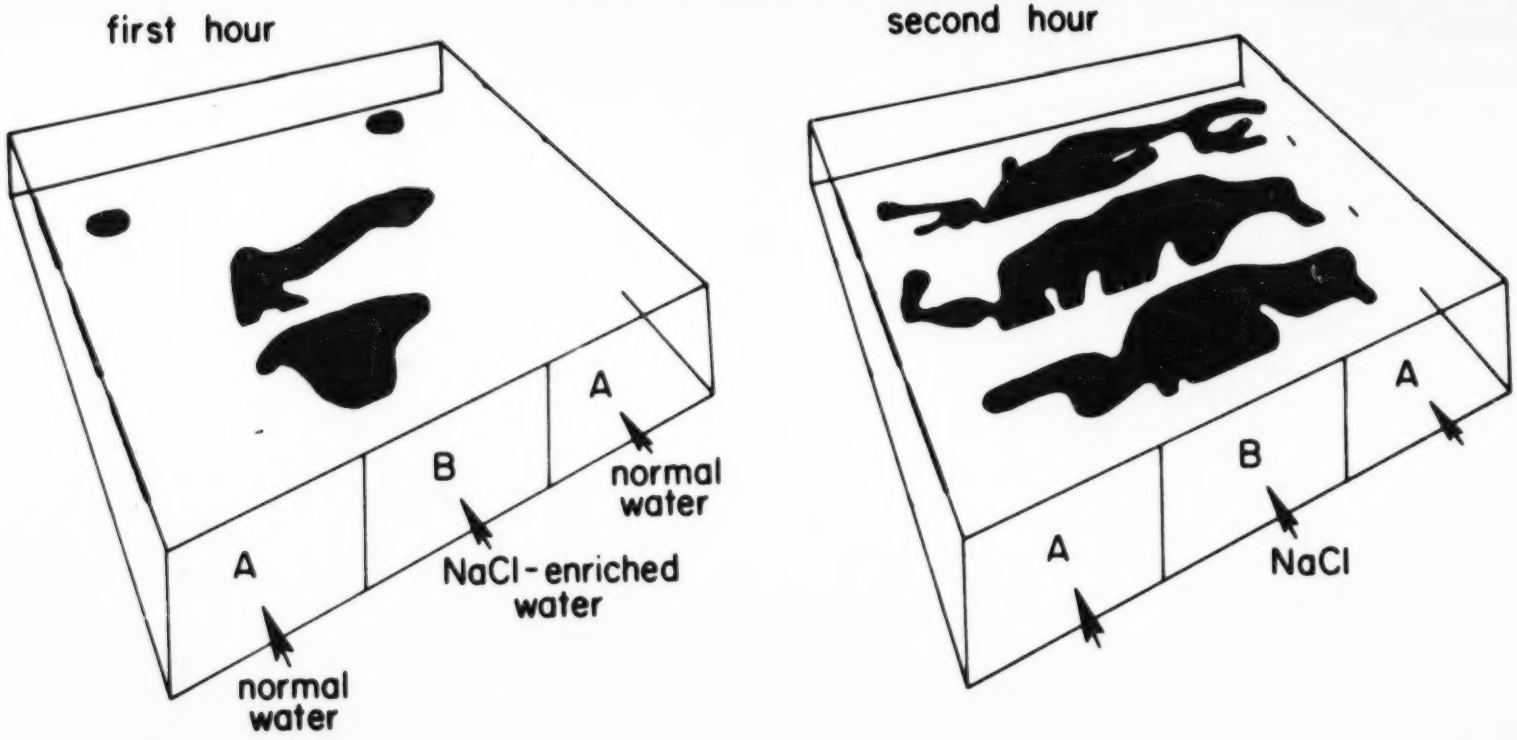
Figure 6

Distributions of Sodium Chloride in the Tank 1 and 2 Hrs. After the Start of the Infusion


first hour
A
B
A
normal water
NaCl-enriched water
normal water
second hour
A
B
A
NaCl

Other Experimental Procedures: Seven experiments were done, each with one naive goldfish, 30 cm long, purchased from a commercial grower in Central Texas. Prior to the experiments, the fish had been kept in the laboratory in large holding tanks in water at 18-20°C which continuously recirculated through filters of activated carbon. The fish were fed with "trout chow" while in the holding tanks, but not in the monitor tank. Each of the seven experiments referred to in this paper consisted of three consecutive eight-hour long recordings of the locomotion of a single fish as follows: 1. with "normal" or "clean" water in the entire tank to account for any effects of transfer to the monitor tank, for behavior peculiar to an exploratory locomotor phase (Kleerekoper et al., 1970), and of time of sojourn in the tank; 2. with NaCl solution infused into the supply section B of the channel so as to make a chloride concentration equivalent to that of a solution of copper chloride containing 50 µg/l Cu^{++}; and 3. with $CuCl_2$ solution infused into section B so as to make a concentration of 50 µg/l Cu^{++} in the supply water of that section of the channel. Note that the maximum possible concentration of copper as $CuCl_2$ was 50 µg/l found only at the boundaries formed by the water exiting through the perforations in the baffle (Figure 5) of section B and the water in the tank. Beyond these points the concentration decreased, particularly at the interfaces with the "clean" water which bounded the mass of advancing chloride-polluted water. Calculations based on the actual electrolytic conductivity profiles indicate that in large proportions of the mass of polluted water the concentration did not exceed 50 µg/l Cu^{++} with very shallow gradients leading to the clean water.

At the high electrolytic conductivity of the laboratory water, even the maximum concentration of copper present in these experiments had not caused mortality in controls after 30 days of exposure in a separate test. The incipient lethal level (Fry, 1947) was not determined.

Treatment of Data: Changes in the locomotor behavior of the fish, however subtle, are reflected quantitatively in some or all of its locomotor characteristics (Kleerekoper et al., 1969, 1970). This fact was used: 1. to ascertain whether exposure to $CuCl_2$, as compared to NaCl of the same concentration, modified the locomotor behavior of the fish; 2. to verify whether such modifications in locomotor behavior were quantitatively related to the spatial distribution pattern of Cu^{++} ions in the tank; 3. to establish whether the modifications resulted in a change in the orientation of the movements and, if so, whether that change was correlated with the spatial distribution of the copper ions; 4. to establish the effects of transfer of the fish to the monitor tank, exploratory behavior, and time in the absence of any "pollutants".

The locomotor characteristics considered in this study were number of events (frequency of photocell responses triggered by presence of fish); time accumulated in an area; distance accumulated; average

velocity; number of turns; average size of turn; variance of size of turn; and heading or orientation of movements.

Changes in any one of these characteristics were investigated by the following statistical techniques: 1. fitting of empirical polynomial models for each characteristic; 2. testing of equality of such models over the different treatments, and 3. an interpretation of the differences in the models, provided that the hypothesis of model equality was rejected.

The data for a particular characteristic, say Z, consisted of observations, Z_{ij}, for each point of a 7 x 7 grid of equidistant X_i and Y_j photocell coordinates representing locations in the tank. Table 2 illustrates the data by listing the observations concerning a single locomotor characteristic for a representative fish under the NaCl and $CuCl_2$ treatments. Preliminary analysis of all data sets by two-dimensional orthogonal polynomials suggested that in most cases a fifth order polynomial was statistically necessary and sufficient to adequately describe the surfaces. The resultant equation is:

$$Z_{ij} = B_{00} + B_{10} + B_{01}Y_j + B_{20}X^2_i + B_{11}X_iY_j + B_{02}Y^2_j + \ldots B_{05}Y^5_j + \varepsilon_{ij}$$

where ε_{ij} denotes the error term. The ordinary least squares procedure was used to provide the best estimates of the B_{ij} coefficients. Table 3 contains both the B_{ij} estimates for the data of Table 2 and a goodness-of-fit criterion.

The polynomial models, besides describing the data, were then used to test the linear hypothesis that a NaCl model equalled the corresponding $CuCl_2$ model. The hypothesis was tested by an F Statistic from the residual sums of squares (see e.g. Ostle, 1963, p. 201). Table 3 supplied the necessary additional statistics for the representative data above.

Upon rejection of the hypothesis of model equality, the third objective was to identify the source of the inequality. The data were paired to block out the effect of location of response; thus, a differential response was determined for each location by subtracting the Z_{ij} observations between treatments. Since the treatment was concentrated in only 25 of the 49 locations, the W_{ij} differences of these 25 "treated" locations were compared with the 24 "control" locations by the Wilcoxon rank sum statistic (Wilcoxon and Wilcox, 1964). This nonparametric statistic tested the hypothesis that the mean difference in the treated region, say u_1, equals the mean difference in the control region, say u_2. Rejection of $H_0: u_1 - u_2$ established that the inequality of models was, at least partially, due to the treatment.

Abundant time series data were also available on the absolute headings of each fish, indeed the total number of headings recorded

Table 2. Numbers of events* on the X_i, Y_j photocell coordinates for experiment 172 in each of the 49 areas of the tank.

		X_i						
		1	2	3	4	5	6	7
		Under NaCl						
Y_j	1	58	35	29	39	42	40	72
	2	20	8	11	7	19	14	19
	3	26	17	10	9	25	18	32
	4	21	20	20	17	22	26	27
	5	13	20	15	13	14	11	27
	6	9	14	7	10	2	13	15
	7	29	44	73	31	35	49	38
		Under $CuCl_2$						
Y_j	1	34	29	21	24	23	16	42
	2	22	19	8	10	18	15	23
	3	25	30	10	16	17	18	24
	4	20	26	21	11	10	7	20
	5	17	20	18	17	19	13	27
	6	9	12	8	18	5	6	20
	7	42	14	27	27	26	24	40

*Numbers of times any of the photocells of one area are triggered.

Table 3. Estimates of polynomial coefficients (B_{ij}) and goodness of fit tests for number of events in experiment 172.

	Estimates for individual treatments		Estimates for combined treatments
	NaCl	$CuCl_2$	
B_{00}	24.18	19.76	21.97
B_{10}	0.93	-0.08	0.42
B_{01}	-0.65	-0.26	-0.45
B_{20}	0.94	1.09	1.02
B_{11}	-0.28	0.13	-0.08
B_{02}	2.90	1.14	2.02
B_{30}	1.12	1.74	1.43
B_{21}	-0.56	-0.10	-0.33
B_{12}	0.08	0.11	0.06
B_{03}	0.86	1.36	1.11
B_{40}	-0.00	0.30	0.15
B_{31}	1.35	-0.17	0.59
B_{22}	0.00	0.11	0.06
B_{13}	-0.29	-0.22	-0.25
B_{04}	1.70	0.72	1.21
B_{50}	-0.05	0.44	0.19
B_{41}	-0.27	0.14	-0.07
B_{32}	0.31	-0.28	0.02
B_{23}	-0.56	0.24	-0.16
B_{14}	0.06	-0.10	-0.02
B_{05}	-0.04	0.19	0.08
Total SS	11901.4	3689.1	71434.8
Residual SS	1312.2	857.1	60931.0
F. statistic	11.30	4.63	0.66

varied from roughly 2,000 to 6,000. The frequencies of individual headings were summarized for each fish with a 2 x 16 table according to the treatment (NaCl vs. $CuCl_2$) and consecutive orientation classes of 22 1/2 degrees each. Table 4 illustrates for experiment 172 the data as produced by the on-line computer.

Since exact angle resolution was not attained (in present experimentation), the angle classes are not equally probable; however, subsequent tests are not biased by this fact. A χ^2 statistic was used to test these multinomial distributions for homogeneity of the populations of headings under NaCl and under $CuCl_2$.

Upon rejection of the homogeneity hypothesis, a series of subsequent χ^2 statistics identified which particular class or classes of headings differed significantly. For each fish, each class was analyzed to determine whether its proportion in the NaCl population of headings equalled its proportion in the $CuCl_2$ population.

Assuming now that the seven fish constitute a random sample from a larger population of fish, the sign test indicates whether the population as a whole prefers a specified orientation under one treatment relative to that under the other. In the absence of such effect, the observed relative frequency of u will exceed that of v with probability 0.5 Hence if the relative frequency of u exceeds that of v for all seven fish there is evidence (at $\alpha = .01$) that the population prefers the specified orientation to that under u relative v.

Table 4. Frequency of heading class v. treatment for fish in experiment 172.

Treatment	Classes of Headings in Degrees							
	0-22	23-45	46-67	68-90	91-112	113-135	136-157	158-180
NaCl	195	35	84	36	120	29	81	53
$CuCl_2$	104	42	62	37	74	25	89	43
	181-202	203-225	226-248	249-270	271-292	293-315	316-337	338-360
NaCl	181	36	51	39	97	41	79	28
$CuCl_2$	99	27	60	25	138	35	75	33

SECTION VI

RESULTS

The Effects of the Presence of Copper Chloride on the Locomotor Characteristics of the Fish in the Tank as a Whole:

The results of the F tests of equality of the empirical polynomial models demonstrated that, in six of the seven fish tested, all the locomotor characteristics investigated were affected by substitution of the sodium chloride by the copper chloride ($F_{21,56} = 2.2$; $\alpha = 0.1$). In one fish only (experiment #194), a single characteristic, time accumulated in various areas of the tank, remained unaffected by the change in condition.

The Changes in Locomotor Characteristic in Region B Resulting from the Displacement of Sodium Chloride by Copper Chloride:

The results of the Wilcoxon rank sum test on these changes are represented in Table 5 for seven naive fish. Increases and decreases in the values resulting from the introduction of the copper ion into that region are indicated by + and −; NS means that the changes are not significant. As sodium chloride was being replaced by copper chloride, the locomotor changes were statistically consistent for three of the characteristics analyzed: Time accumulated, average size of turn, and variance of the size of turn; all three increased highly significantly in, respectively, four, five, and four of the seven fish studied. In the remaining fish the test yielded nonsignificant results. Throughout, three fish (experiment nos. 172, 190, 194) behaved consistently with respect to all characteristics analyzed, whereas, one fish (no. 173) showed inconsistency in only one characteristic. Entirely inconsistent were the data from experiments nos. 197 and 198.

Changes in the Orientation of Locomotion in the Tank as a Whole in Response to the Displacement of Sodium Chloride by Copper Chloride:

In all fish, the frequency distribution of all the headings during copper chloride exposure was significantly different from the distribution during sodium chloride exposure (Table 6). The analysis of individual headings reveals that, under copper chloride, in five of the seven fish the observed relative frequency of headings in the 226–248° arc significantly exceeded the corresponding frequency under sodium chloride. Since the other two fish also exhibited the same phenomenon (though not significantly), it is clear ($\alpha = .01$) that this characteristic is valid also for the population as a whole. For no other angle category was this deviation consistent in the whole sample of seven fish. That the phenomenon might extend to the whole arc between 225–293° is suggested in the binomial distributions of sixteen headings during exposure to sodium chloride (two hours) for all seven fish.

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Table 5. Test of the Wilcoxon rank sum statistic of changes in locomotor characteristics in region B of tank resulting from exposure to $CuCl_2$; +, increase; -, decrease; NS = not significant at $\alpha = .05$

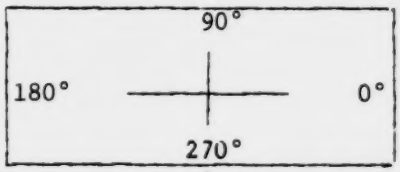
	Experiment no.						
Characteristic	170	172	173	190	194	197	198
No. of events (frequency of photocell responses	+	+	+	+	+	-	-
Time accumulated in region B	+	NS	+	+	NS	+	NS
Distance accumulated	+	+	+	+	+	-	-
Average velocity	NS	NS	-	NS	NS	NS	+
No. of turns	+	NS	+	+	NS	NS	-
Average size of turn	+	+	+	NS	NS	+	+
Variance of size of turn	+	+	+	+	NS	NS	NS

SECTION VII

DISCUSSION

The goldfish in these experiments perceived the copper ions present in the "polluted" region of the tank at the prevailing concentration of 5 µg/l and they responded with modification of their locomotor characteristics in the tank as a whole as demonstrated by the analysis of fitted surfaces. Within the polluted region, the orientation of their movements deviated significantly in upstream direction as compared to their orientation in NaCl of equivalent concentration. This behavior resulted in an increase in time spent by the fish in the copper-affected region. Since velocity was not affected in five of the seven experiments and decreased in only one there is no evidence for a stupefying effect of the copper ion on the fish (Jones, 1964). Furthermore, the total distance swum in the polluted region actually increased for all the fish and the number of entries into the polluted region increased for five of the seven fish, (Table 5). Thus, the analysis must lead to the conclusion that copper chloride, at the concentration which prevailed in the polluted region, was either "attractive" to the goldfish or led to the entrapment of the animal. The nature of the phenomenon becomes evident by an analysis of the orientation behavior of the fish (Table 6, Figure 7). For the sample of seven fish the positive deviations of the frequency distribution of headings during the copper chloride exposure in comparison with the same distribution in sodium chloride were in the 226-294° sector, that is, in the general upstream direction in the tank, with no consistent deviations in any of the remaining headings. A separate analysis of only the downstream movements established that the deviations of their headings were distributed at random. The interpretation of these results is that the copper, in the conditions of the experiment, released in the animals positive rheotaxis which was accompanied neither by increased velocity nor increased turning frequency, changes which are frequently associated with "alarm response". Distance, on the other hand, increased in five of the seven experiments. Thus, the significant and consistent preference for upstream swimming during the copper pollution resulted in more extensive cruising and increased time expenditure within the copper-affected region, as if the fish had become entrapped there. The conclusions drawn seem to be at variance with those of some other studies of the locomotor response of fish to solutions of metal salts (Jones, 1947,1948; Sprague, 1963, 1964, 1968; Ishio, 1965; Burden, 1945; Hill, 1956; Syazuki, 1964; Saunders and Sprague, 1967; Sprague et al., 1965; Sprague and Ramsay 1965; Grande, 1967) which pointed to "avoidance" behavior, although a few studies failed to show avoidance of heavy metal solutions (Summerfelt and Lewis, 1967); in one instance "attraction" to a high copper sulphate solution was reported (Jones, 1964). In the latter case, the behavior was attributed to the stupefying effect of the pollutant. It is to be noted,however, that the results reported here and those in the literature are not readily comparable. The experimental

Table 6. Significant deviations in headings following the change



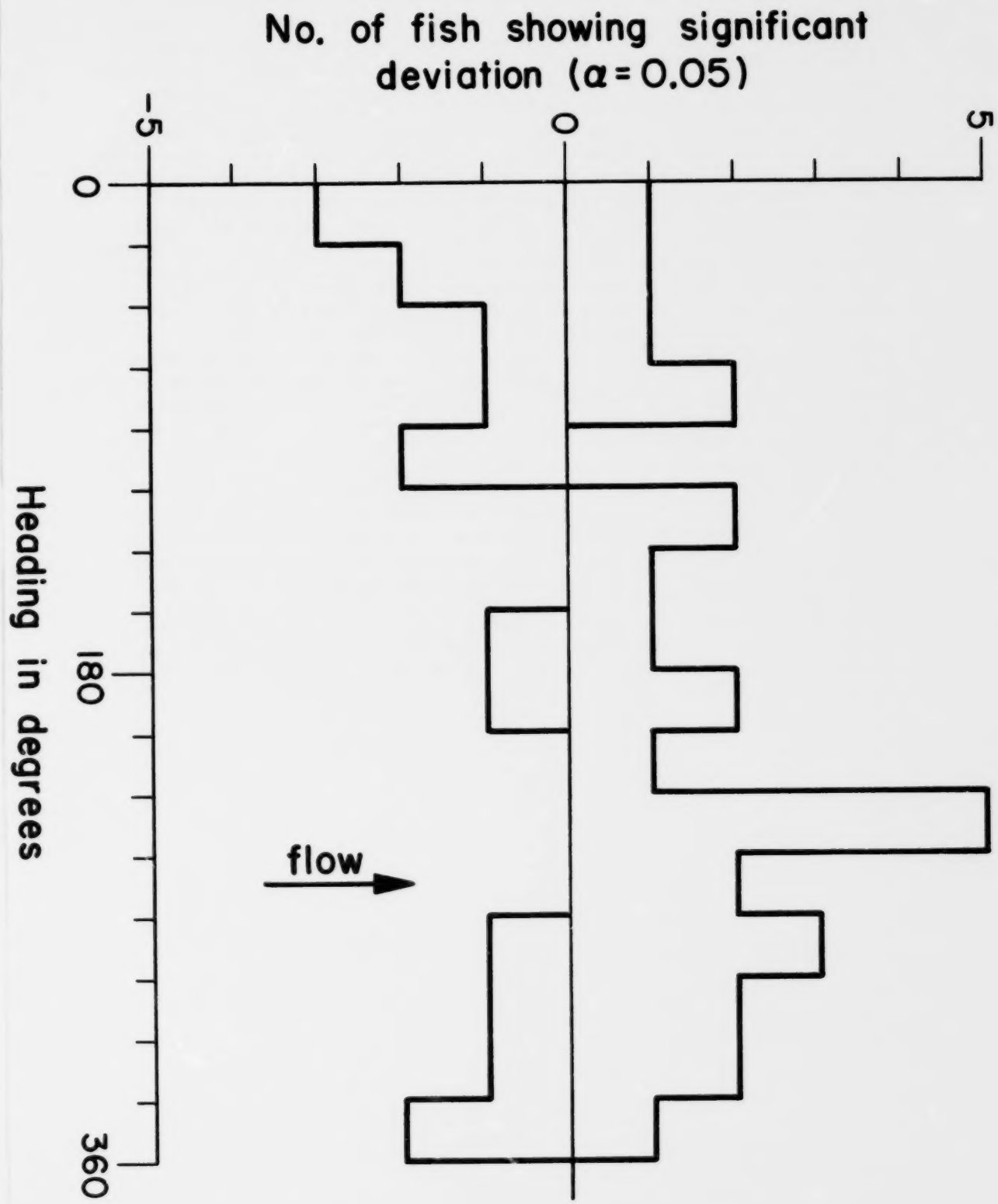
No. of experiment	Headings in degrees							
	0–22	23–45	46–67	68–90	91–112	113–135	136–157	158–180
170	+	–	–	–	NS	NS	NS	NS
172	–	NS	NS	NS	NS	NS	+	NS
173	NS	NS	NS	NS	NS	NS	NS	NS
190	–	NS	NS	+	NS	NS	NS	NS
194	NS	NS	NS	NS	NS	NS	NS	NS
197	NS	–	NS	NS	–	+	NS	–
198	–	+	+	+	–	+	NS	+

of exposure from sodium chloride to copper chloride ($\alpha = 0.05$)

181-202	203-225	226-248	249-270	271-292	293-315	316-337	338-360
+	NS	NS	NS	+	NS	NS	NS
-	NS	+	NS	+	NS	NS	NS
NS	NS	+	NS	+	-	-	NS
NS	NS	+	NS	NS	NS	NS	-
NS	NS	NS	+	NS	+	+	-
+	NS	+	NS	NS	NS	NS	NS
NS	+	+	+	-	+	+	+

Figure 7

Binomial Distribution of the Significant Deviations of Headings on Exposure to $CuCl_2$ as Compared with NaCl of the Same Chloride Concentration.


No. of fish showing significant deviation (α = 0.05)
-5
0
5
0
180
360
Heading in degrees
flow

techniques used in the present study were not only very different from those applied to the problem by other workers but the fish were in an open field situation with a very shallow gradient as opposed to the conditions prevailing in gradient tubes and troughs. Furthermore, the data permitted a much more detailed analysis of locomotor behavior than had been possible hitherto. In addition, differences in species (Ball, 1967), hardness of the water (Cairns and Scheier, 1957; Tarzwell and Henderson, 1960; Lloyd, 1960, 1961; Lloyd and Herbert, 1962; Screenivasan and Soundar, 1963; Herbert and Wakeford, 1964), other aspects of water quality (Doudoroff and Katz, 1953; Jones, 1939; Lloyd and Herbert, 1962; Mount, 1966; Malacea and Gruia, 1964), the interaction and additive effects of various ions (Southgate, 1932; Friedland and Rubleva, 1958; Bucksteeg et al., 1955; Cherkinsky, 1957; Herbert, 1962; Herbert and Shurben, 1964; Brown et al. 1969), and acclimation (King, 1937; Paul, 1952; Afleck, 1952; Grande, 1967; Lloyd, 1960; Pickering and Vigor, 1965; Schofield, 1965) are all known to be important variables affecting the physiological and behavioral responses to heavy metal pollution. Few of these variables have been studied in any detail and the results of their interactions are largely unknown. Should the locomotor behavior of the goldfish, in response to sublethal concentrations of copper ions in an open field, be representative of that displayed by other species, the phenomenon described might be of considerable importance for the assessment of the biological effects of copper pollution. In the field, the behavior described might lead the fish towards an upstream source of pollution, hence, into increasingly, higher concentrations of the metal. At a critical concentration,dependent on species and water quality, avoidance reactions (Sprague, 1963, 1964; Sprague et al., 1965) might then replace the orientation behavior described here. However, the response to a potentially harmful concentration, reached through a shallow gradient, may be different from that elicited by a steep gradient.

SECTION VIII

THE LOCOMOTOR RESPONSE OF THE GOLDFISH, CHANNEL CATFISH AND LARGEMOUTH BASS TO A "COPPER-POLLUTED" MASS OF WATER IN AN OPEN FIELD

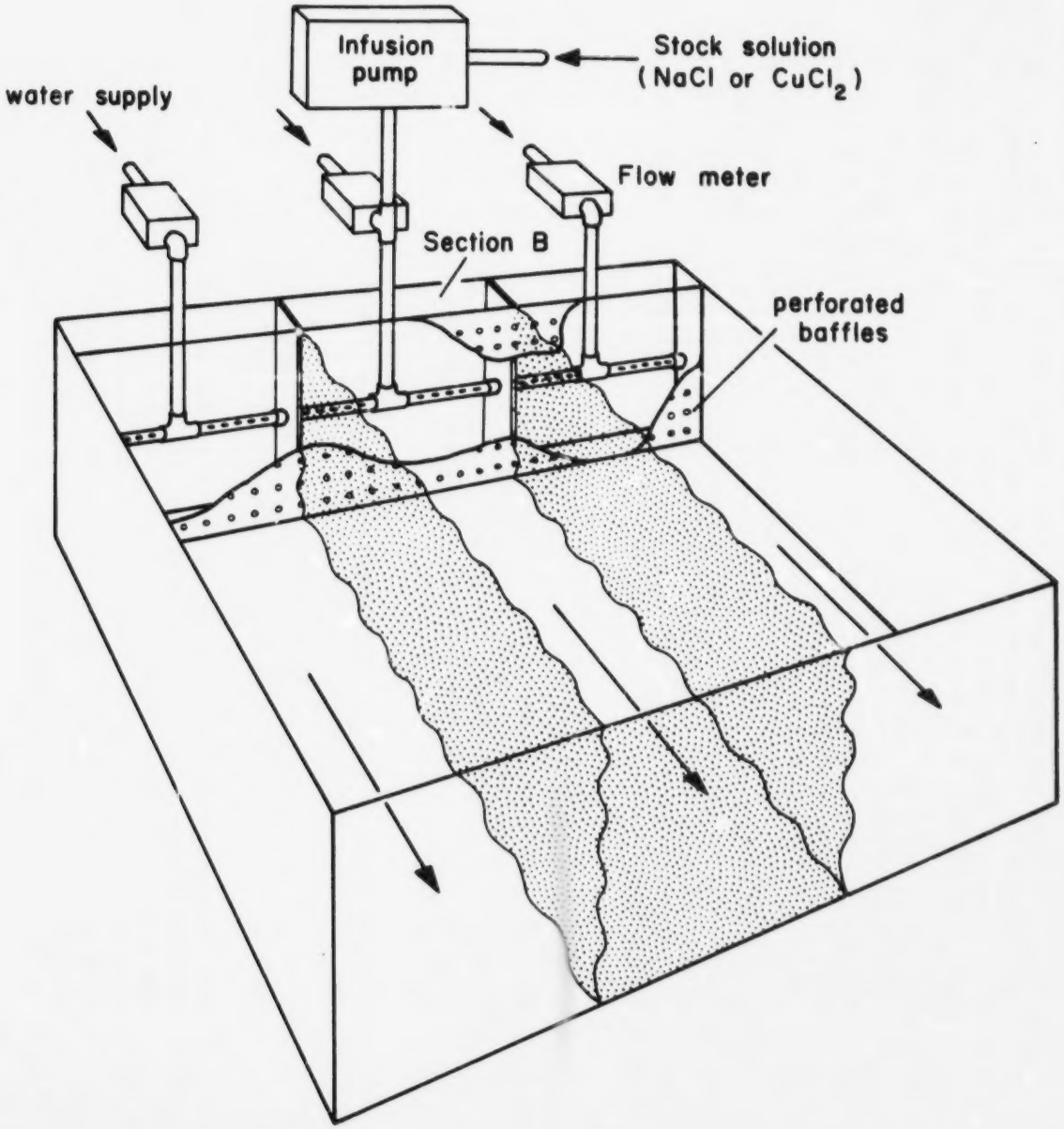
The previous experiments established that in an open field, the locomotor behavior of goldfish changed on entering a shallow gradient of copper ions. The orientation of the fish in the copper-containing water deviated consistently in the upstream direction and was due to the copper rather than to the chloride ion. The concentration in most of the mass was 11-17 µg/l with isolated pockets containing maximally 50 µg/l Cu^{++} as $CuCl_2$. As stated, these results are at variance with those reported in the literature (Burden 1945; Grande 1967; Hill 1956; Ishio 1965; Jones 1947, 1948; Sprague 1963, 1964, 1968; Saunders & Sprague 1967; Syazuki 1964; Sprague & Ramsay 1965; Sprague et al., 1965) indicating distinct "avoidance" behavior, when the fish are exposed to a steep gradient of copper ions within a relatively restricted space.

The following experiments were designed to ascertain: 1. the behavior of the fish when exposed to a steep gradient of copper ions in an open field situation, and to determine whether, in such conditions, "avoidance" behavior could be elicited; 2. the locomotor behavior of the fish within a mass of water containing uniformly distributed copper ions without possibility of "avoidance". The experiments were done in monitor tank #1. The size of the perforations in the baffles of the supply channel (B in Fig. 5) was increased resulting in a different flow pattern and slope of the copper gradient. As before, solutions of sodium or copper salts were delivered from a supply vessel through silastic tubing and stainless steel fittings by a chemical feeder into the water pipe (PVC) supplying the central 1/3 of the inlet channel (Fig. 5). When the chemicals were introduced into section B only, the diluted solution traversed the main body of "clean" water forming boundary diffusion gradients whose characteristics were verified by determining the three-dimensional distribution of the ions in the tank as described above (Section IV). The sodium or copper salt-enriched water introduced into section B moved across the tank as a mass whose boundaries are indicated in figure 8. During the first two hours the ions affected about 1/3 of the mass of the tank water. Because of the very distinct definition of that volume of water affected by the ions (region B) and that unaffected (region A) after two hours of recording, locomotor characteristics in these two regions could be accurately analyzed and compared during that period. After the initial two hours the copper ion gradually contaminated the whole water mass of the tank.

Thirty-four experiments were done, each with a naive fish. Twenty of the test fish were goldfish (Carassius auratus), 28-30 cm long and obtained commercially in Texas, seven were largemouth bass (Micropterus salmoides), 28-30 cm long, and seven were channel catfish (Ictalurus

Figure 8

Three-dimensional Representation of Monitor Tank
Showing the Advance of the $CuCl_2$ Boundary


Infusion pump
Stock solution (NaCl or $CuCl_2$)
water supply
Flow meter
Section B
perforated baffles

punctatus), 32-35 cm long. The latter two species were obtained from the State Fish Hatchery at Huntsville, Texas. The goldfish were held, prior to experimentation, in large community tanks and the bass and catfish were kept individually in smaller tanks. The water was 18-20 degrees C and was recirculated through carbon filters. The goldfish and catfish were fed "trout chow" and the bass live minnows. Feeding was daily except when the fish was in the monitor tank. Each experiment consisted of three consecutive parts: 1. a twenty-four hour recording period with laboratory water; 2. an eight hour period with NaCl solution infused into the supply section B so as to make a chloride concentration equivalent to that of a solution of copper chloride containing 50 µg/l Cu^{++}, and 3. an eight hour period with $CuCl_2$ solution infused into section B so as to make a concentration of 50 µg/l Cu^{++}. The concentration of copper as $CuCl_2$ was 50 µg/l only at the boundaries formed by water exiting from section B and the surrounding water. Beyond these points the concentration decreased. The concentrations of ions exiting section B was empirically verified by atomic absorption analyses. Ten goldfish, seven bass and seven catfish were tested in this manner. In addition, ten goldfish were tested under a regimen of 100 µg/l. The chemical characteristics of the water supply to the monitor tank are indicated in Table 1.

Treatment of data: The analyses of locomotor behavior were those as described (Section IV) with some additions. The locomotor characteristics considered were: activity as indicated by the number of events (frequency of photocell responses); time accumulated in an area; distance accumulated; average velocity; number of turns; average size of turn; variance of turn size, and heading or orientation of movements.

Changes in any of these characteristics were expressed by the statistical techniques described and summarized as follows:

1. An F test of the linear hypothesis that a polynomial model for NaCl data equalled the corresponding model for $CuCl_2$ data.

2. If this hypothesis was rejected, the source of the inequality was tested using the Wilcoxon rank sum test. The NaCl response was subtracted from the $CuCl_2$ response for each photocell, thereby minimizing effects of location, and the differences in region A compared with those in region B. This test of mean differences between the two regions, if rejected, establishes that the inequality of the models is, at least partially, due to the treatment.

3. A χ^2 statistic was used to test the multinomial distributions of headings, in 20 degree categories, for homogeneity of the populations of headings under NaCl and under $CuCl_2$. Upon rejection, a series of subsequent χ^2 statistics identified the particular classes of headings differing significantly. A sign test ascertains whether the

population as a whole prefers a specified orientation under one treatment relative to the other.

In addition to these procedures the following analyses were made:

1. An analysis of exploratory behavior as defined by Kleerekoper et al. (1972) which essentially determines, on a temporal scale, the changes in location of a center of gravity of locomotion by computing, for each sequential time period, the mean and variance of the mean x and y coordinates of the animal's locomotion in the monitor tank.

2. An analysis of the orientation response to copper ion by means of an analysis of variance: each data set was grouped into a frequency distribution with 18 angle categories of 20° interval width. Two such distributions were obtained for each fish; the first summarized orientation over a two-hour control period whereas the second applied to a subsequent two-hour period of copper treatment. Let X_i; i = 1,..., 18; denote the observed frequencies in the control period and Y_i; i = 1, ..., 18; the frequencies during the copper treatment. Inasmuch as the X_i and Y_i categories are unequally probable in a bounded square grid, the data were transformed according to

$$Z_i = (Y_i - X_i) / (Y_i + X_i).$$

Conceptually the Z_i variates, which can range from -1 to +1, represent an index for the preference of angle category i under the treatment and have expectation 0 for each angle category under the null hypothesis of the absence of a treatment effect.

This analysis was performed on 28 goldfish with two groups of ten randomly chosen for the 50 µg and 100 µg copper treatment and eight remaining under control conditions (0 µg) to detect possible time trends. A two way factorial analysis of variance analyzed the data to detect differences in the mean response of the fish, the angles, and/or their interaction. The fish variation was partitioned into four independent sources; among treatment concentrations and within each of the three concentrations (0,50, and 100 µg). Preliminary scanning revealed an apparent correlation between angle categories 180° apart. Therefore, defining a "track" as an angle category and its 180° counterpart, the variation among angles was partitioned into a variation among the nine tracks and within each of the nine tracks. Finally, the above partitionings suggested dividing the interaction sum of squares into the 40 components obtained by crossing the four sources of fish variability by the ten sources of angle variability.

3. A comparison of locomotor behavior during a two hour control period and the final two hours of $CuCl_2$ introduction, when the chloride had become distribututed throughout the monitor tank, to analyze changes brought about by the fish finding itself in contaminated water from

which it could not escape. The locomotor parameters utilized in this aspect of the study were those itemized earlier (Kleerekoper et al. 1970).

Results: The results refer to two experimental conditions: I. the fish could move freely between the zone affected by copper chloride (region B) and that containing sodium chloride of the same concentration (region A), and II. all the water in the monitor tank contained copper chloride and, hence, there was no free choice for the fish.

I. Exposure to either copper chloride or sodium chloride by free choice.

1. Polynomial Model

The results of the F test of equality of the empirical polynomial models demonstrated that in all of the 34 fish tested, all the locomotor characteristics investigated were affected by substitution of the sodium chloride by the copper chloride ($\alpha = 0.1$).

Two of the seven bass, three of the seven catfish and seven of the ten goldfish showed a consistently non-significant difference in all locomotor parameters in region B following the displacement of the sodium chloride by 50 µg/l of copper chloride (see methods, Wilcoxon rank sum test, $p. < .05$). The remaining individuals tended to increase (+) the number of events, the time spent, the distance swum and the number of turns made in region B relative to region A (Tables 7 and 8).

The same technique applied to ten goldfish tested at 100 µg/l of copper ion indicated that four individuals were unaffected. The other six goldfish were inconsistent, but two of them tended to increase time spent, distance swum and number of turns made in the mass of water containing the copper ion (Table 7).

2. Exploratory Behavior

The presence of the copper ion altered the center of gravity of locomotion in the tank as a whole as specified by the analysis of exploratory behavior (see methods). Six of the seven bass, all of the catfish, and five of the ten goldfish displayed a significant change in either the mean of variance of the x or $\bar{y}$ coordinate in 50 µg/l of copper ion. Five of the ten goldfish tested at 100 µ/l were significantly affected.

3. Headings (Orientation in the tank as a whole)

A comparison of the headings between the exposure to sodium and copper ions (see methods) for the three species tested at 50 µg/l indicated that for the bass there was no change in the distribution, for six

of the seven catfish there was a significant increase in the 100° - 170° arc, and for eight of the ten goldfish a significant increase in the 110° direction. Eight of the ten goldfish tested at 100 µg/l increased the number of headings in the copper in the 10° direction.

The analysis of variance performed on the 18 headings (nine tracks) tabulated for goldfish in control periods (0 µg/l, eight fish), in 50 µg/l (ten fish) and in 100 µg/l (ten fish) revealed that all sources of variation were significant ($p < .01$, Table 9). The mean index for each track in each of these groups is shown in Figure 9. The overall mean index did not change between controls (1305 $\pm$ .0510) and 50 µg/l (.1294 $\pm$ .0375), but was significantly less (-.0076 $\pm$ 0.0704) in 100 µg/l (Wilcoxon rank sum test, $p < .05$, Figure 10). The partitioning of the main sources of variation into their components indicated that in 50 µg/l three tracks (Figure 11, broken lines) were significantly increased compared to those in the sodium ion, forming an arc from 140° - 220° and 320 - 40° (resultant shown as heavy black line). In 100 µg/l the only track which changed significantly was composed of the 50° and 230° directions. In all cases the size of the index in either component of the track was not significantly different. The dispersion among tracks was greater in the 100 µg/l group than in either of the other two (Figure 10).

II. Exposure to copper chloride without choice

The effects of this condition were analyzed by comparing the data from the seventh and eighth hours of the initial control periods and the final two hours of copper ion introduction which were the corresponding seventh and eighth hours of recording after the commencement of the copper chloride introduction into the monitor tank. By this time the ion had been carried by the water flow completely across the tank to the exit side. Only turning behavior was altered significantly. The mean angle size of turn decreased in two and increased in three of the five goldfish tested at 50 µg/l but it increased in all five goldfish tested in 100 µg/l. Accordingly, these latter individuals consistently increased the number of smaller-sized angles of turns.

TABLE 7

Wilcoxon Rank Sum Test (significant at $p < .05$)
Each column represents an individual which showed a significant change

PARAMETER	Bass (50 µg)					Catfish (50 µg)			
No. of events	+	+	-	+	NS	+	NS	NS	NS
time accumulated	NS	+	NS	+	NS	+	+	NS	NS
dist. accumulated	+	+	-	+	NS	+	NS	NS	NS
average velocity	+	NS	-	NS	+	NS	-	NS	+
no. of turns	+	+	-	+	NS	+	NS	NS	+
average turn size	NS	NS	NS	NS	+	NS	NS	-	NS
variance of turn size	NS	NS	-	NS	NS	+	NS	-	NS
SUMMARY									
-	0	0	5	0	0	0	1	2	0
+	4	4	0	4	2	4	1	0	2
NS	3	3	2	3	5	2	5	5	5

Goldfish (50 µg)			Goldfish (100 µg)					
+	+	+	NS	NS	+	NS	-	NS
NS	NS	NS	+	NS	+	NS	-	NS
+	NS	+	+	NS	+	NS	-	NS
NS	NS	NS	NS	+	NS	NS	NS	-
+	+	NS	+	NS	+	-	-	NS
NS	NS	NS	NS	NS	NS	NS	NS	-
NS	NS	NS	NS	NS	NS	NS	NS	-
0	0	0	0	0	0	1	4	3
3	2	2	3	1	4	0	0	0
4	5	5	4	6	3	6	3	4

TABLE 8

Summary of results for Wilcoxon rank sum test (50 µg/1)
Number of fish in brackets

PARAMETER	BASS(7)		CATFISH(7)		GOLDFISH(10)		POOLED	
	+	-	+	-	+	-	+	-
no. of events	3	1	1	0	3	0	7	1
time accumulated	2	0	2	0	0	0	4	0
dist. accumulated	3	1	1	0	2	0	6	1
average velocity	2	1	1	1	0	0	3	2
no. of turns	3	1	2	0	2	0	7	1
average turn size	1	0	0	1	0	0	1	1
variance of turn size	0	1	1	1	0	0	1	2

TABLE 9

ANALYSIS OF VARIANCE ($p < .01$)

Source of variation	d.f.	MS
FISH	27	2.1018
Among concentrations	2	1.0300
Within control (0 µg/l)	7	1.6600
Within 50 µg/l	9	0.6255
Within 100 µg/l	9	4.1588
HEADINGS	17	0.0205
Among tracks	8	0.0413
Within tracks	9	0.0021
FISH X HEADINGS	459	0.0270
Among concentrations X Headings	34	0.0185
Within control (0 µg/l) X Headings	119	0.0183
Within 50 µg/l X Headings	153	0.0441
Within 100 µg/l X Headings	153	0.0186

Figure 9

Mean Index of Orientation in Goldfish for Nine Tracks
at 0, 50, and 100 µg/l Cu^{++}

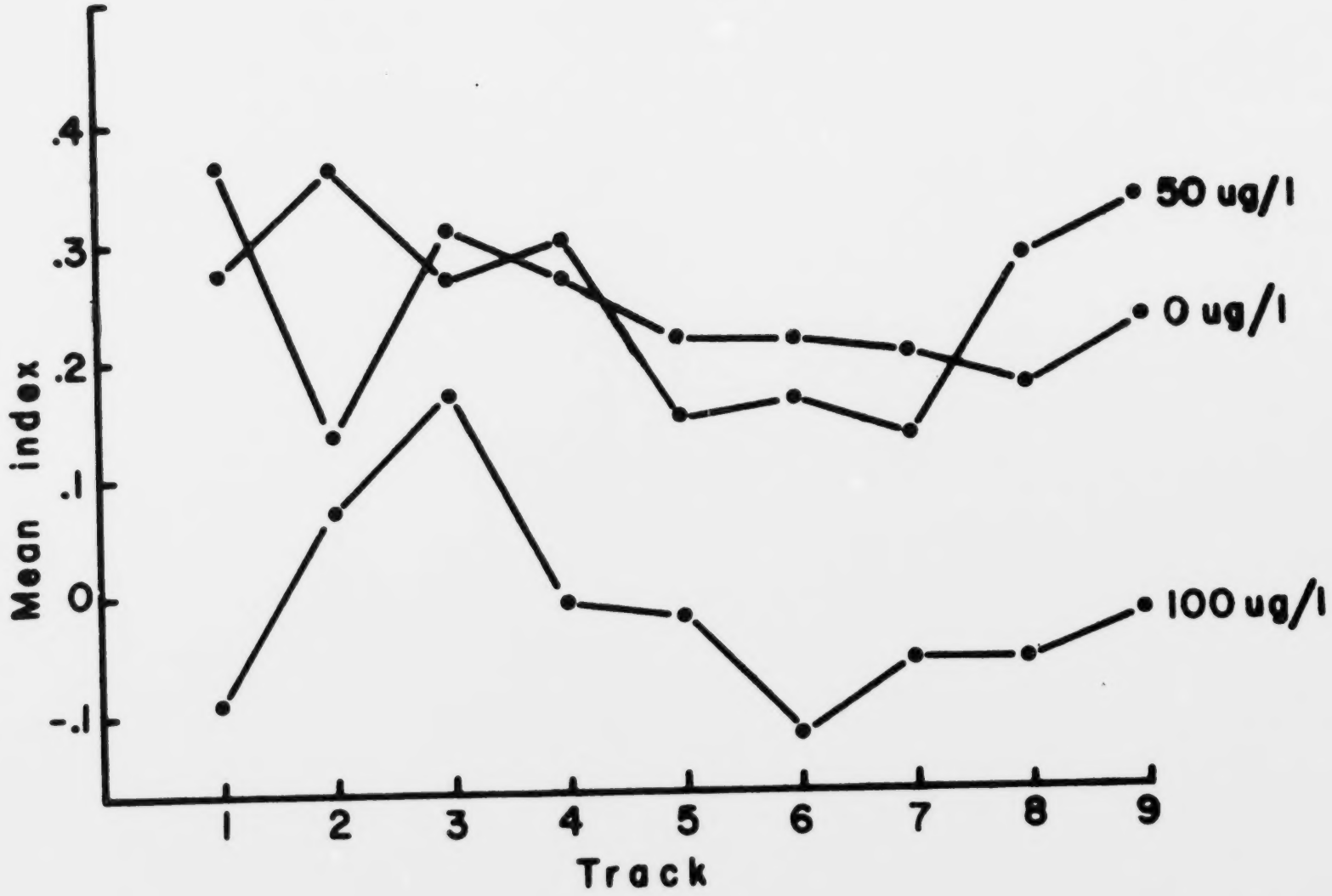

.4
.3
.2
.1
0
-.1
Mean index
1
2
3
4
5
6
7
8
9
Track
50 ug/l
0 ug/l
100 ug/l

Figure 10

Overall Mean Index of Orientation in Goldfish for 0, 50, and 100 µg/l Cu^{++}

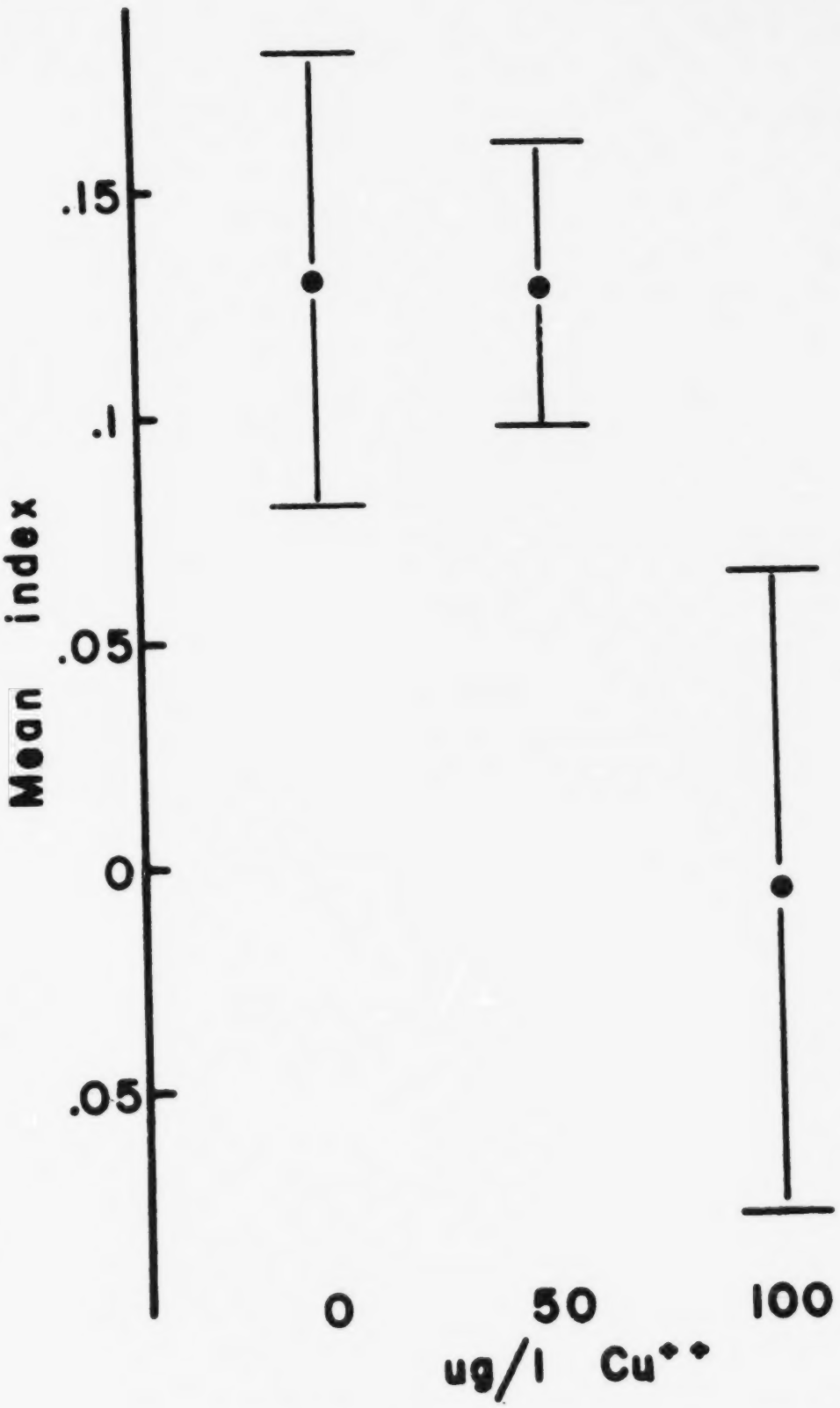
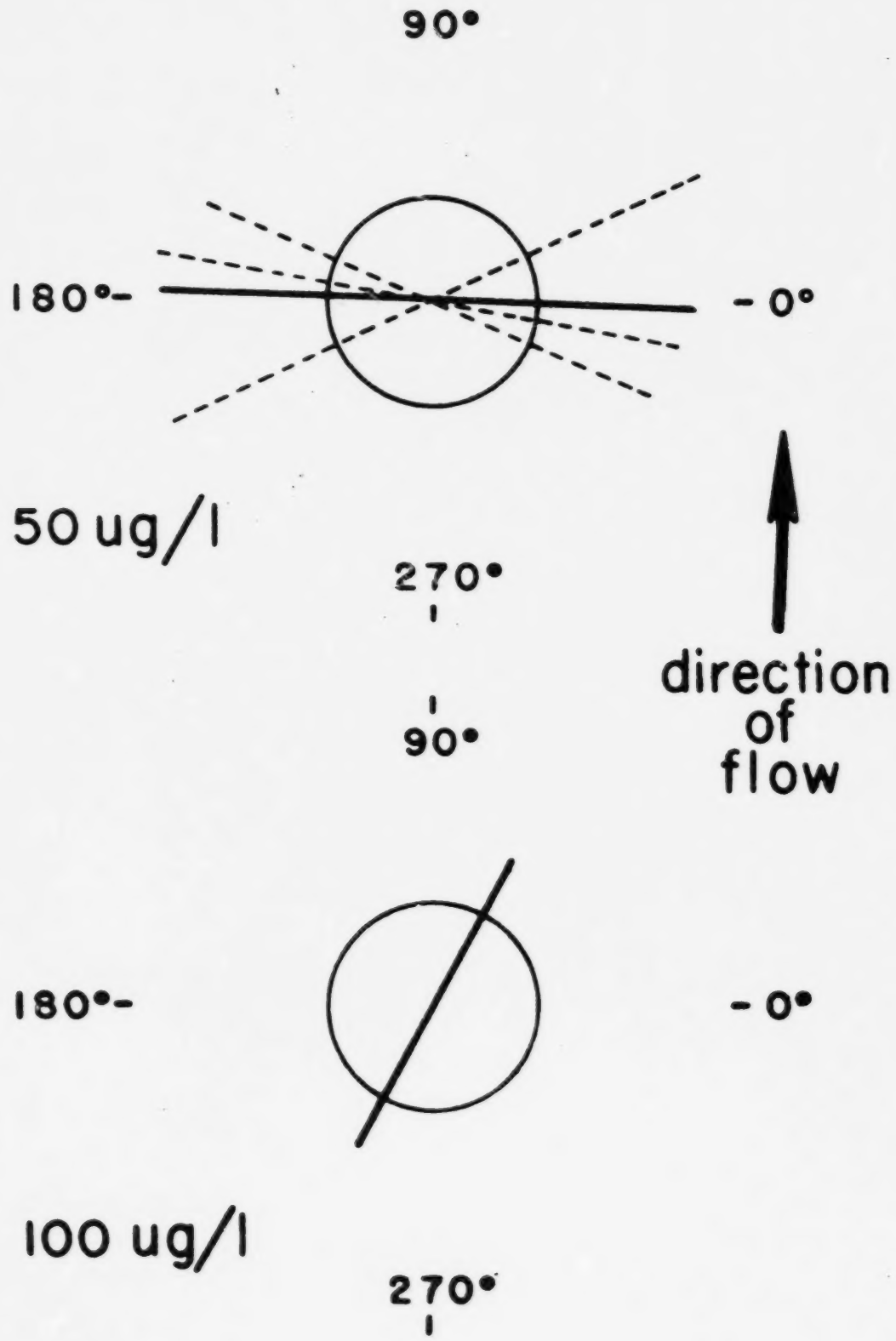

.15
.1
.05
0
.05
Mean index
0
50
100
ug/l Cu++

Figure 11

Orientation in Tracks (Preferences for an Angle Category and its 180° Counterpart) at 50 and 100 µg/l Cu^{++} in Goldfish


90°
180°-
-0°
50 ug/l
270°
90°
direction
of
flow
180°-
-0°
100 ug/l
270°

SECTION IX

CONCLUSIONS

The orientation of none of the largemouth bass was affected by the presence of copper ions at the experimental concentration. However, all but one of the seven catfish and eight of the ten goldfish changed their mean orientation as well as the "tracks" of locomotion highly significantly in the copper-affected region of the tank. The above changes, rather than indicate even a weak degree of "avoidance" of the copper source, point to movement in the direction of the source but to a lesser degree than that in shallower gradients as documented earlier (Kleerekoper et al., 1972). In the goldfish, when surrounded by copper-containing water without access to "clean" water, only turning behavior was affected and no change in orientation could be observed.

It is concluded that the characteristics of the copper gradient between "clean" and "polluted" water rather than the eventual highest concentration of the ion affect the orientation and locomotor behavior generally of the catfish and goldfish.

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SECTION X

THE LOCOMOTOR RESPONSE TO A STEEP GRADIENT OF COPPER IONS BY THE GOLDFISH

The studies reported in the previous sections on the locomotor response by fish to gradients of sublethal concentrations of copper ions in an open field brought evidence for significant changes in orientation in the direction of the copper source.

Furthermore, at the same maximum concentration, the slope of the copper gradient to which the fish were exposed seemed to affect the response. Strong "attraction" toward the copper source in a very shallow gradient, lesser attraction in a somewhat steeper gradient. The aim of the following experiments was to verify whether avoidance behavior might be elicited by confronting the fish with a steep gradient, in a restricted field, in conditions otherwise similar to those which resulted in "attraction".

Methods

The experiments were carried out in monitor tank #2. In the experiments here reported, carbon-filtered water at 21°C was led through glass tubes to the periphery of each of the 16 compartments at a rate of 200 ml/min.

By means of an infusion withdrawal pump, $CuCl_2$ stock solution was infused into the water supply leading to any one compartment at a rate of 0.92 ml/min to produce there the desired concentration of copper. Experiments were done at .005 (7) .01 (9) 0.25 (8), and 10.0 (6) ppm Cu^{++} in addition to controls (5). In all experiments but those at the 0.25 ppm concentrations, a single fish was introduced into the tank once the desired copper concentration in the compartment had been reached. At the 0.25 ppm concentration, the fish entered the tank at the beginning of the introduction of the copper chloride whose full concentration in the compartment was reached in about two hours. Copper levels were monitored by atomic absorption analysis.

In each of the 35 experiments, done between July and September 1971, the movements of a single fish were monitored during eight hours following its release in the tank.

The 22 goldfish (28-30 cm) used in these experiments had been obtained from a commercial grower in central Texas and kept during several months in large holding tanks supplied with recirculating carbon-filtered water. The source and the temperature of this water were the same as those of the water used for the experiments. The fish were fed daily, in the morning, with a dry "trout food" but received no food in the monitor tank.

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The frequency of exposure to copper solutions is shown below.

Number of fish	Number of times fish were exposed to copper ions
12	1
1	2
2	3
6	4
1	5

The discrepancy between the number of exposures and the number of experiments reported is due to the occasional occurrence of periods of inactivity of the fish in the course of an experiment.

Treatment of data

The percent distributions of the frequency of entry and time spent in each compartment were obtained for each experiment. All these distributions were rotated so as to line up to the copper-releasing compartments which were then considered to be the same in the subsequent analyses.

A separate two-way analysis of variance for each variable was used to test the significance of differences between compartments and of the treatment by compartment interaction. The two-way table had slightly varying cell sizes due to a recording failure of 2.5%; hence, the approximate, unweighted means analysis was used.

In order to isolate the copper effect alone, the treatment by compartment interactions was further studied by constructing contrasts. Copper solution was infused into one compartment only but a certain amount of spreading of the copper ion must be expected at the exit of the compartment, prior to the outflow of water through the standing pipe. A copper zone, defined as the copper-releasing compartment and one compartment on either side of it, took into account this lateral spread and hence a table of means was prepared which compared for each treatment level the mean percent entries in the copper zone to the mean of the thirteen remaining compartments. A contrast then tested the interaction of these means over increasing concentrations of copper.

Interactions of the percent time spent variable were studied in like manner. However, since there is little back-flow into neighboring compartments, the percent time spent in the copper-releasing compartment was compared to the mean percent in the fifteen remaining compartments.

Results

In Figure 12 the ratios of the treatment mean to the control mean for the percent compartment entries are illustrated for the copper zone

and the remaining compartments for concentrations of 0.05, .01, .025, and 10.0 ppm. Figure 13 illustrates the ratios for the time spent in the copper compartment and the remaining compartments. With increasing copper concentration the fish entered the copper zone less frequently and spent less time in the compartment into which the copper was infused.

Discussion

The results demonstrate a significant degree of "avoidance" of the copper zone dependent on the copper ion concentration. However, even at the highest concentration tested (10.0 ppm), there was no evidence for an absolute avoidance of the copper-containing compartments. Since both the relative number of entries into the copper zone and the time spent there per entry were affected, it appears that the perception of the copper may either elicit a change in orientation resulting in avoidance of the copper zone or an escape response once the zone has been entered.

In previous experiments carried out at similar copper concentrations and temperature the fish had been kept in the large monitor tank (#1) (5.0 x 5.0m) which allowed for unrestricted locomotion within the confinements of the walls. Unlike the conditions of the experiments here reported, the boundaries of the copper-affected zone consisted of shallow gradients. The fish could swim into and through these gradients in all directions, entering and leaving the copper-affected zone. Under these conditions significant orientation against the current of the copper "polluted" water was observed (Kleerekoper *et al.*, 1972). In the subsequent experiments in a steeper gradient, with goldfish, largemouth bass, and channel catfish the fish still oriented toward the copper source but to a lesser degree (Timms *et al.*, 1972). It is apparent from those results and from the ones here reported that at similar copper concentrations and within a similar temperature range the orientation response is, at least in part, dependent on the steepness of the gradient encountered by the fish. The available information leads to the conclusion that significant "attraction" by copper-"polluted" water can take place when the exposure occurs through a shallow gradient: "avoidance" or "escape" response may result when a similar concentration of copper is encountered through a steep gradient. A gradient of an intermediate slope still "attracts" the fish but to a lesser degree than a steep gradient.

Figure 12

The Ratio of the Treatment Mean to the Control Mean for Percent Entries into Compartments With and Without Copper (Monitor # 2)

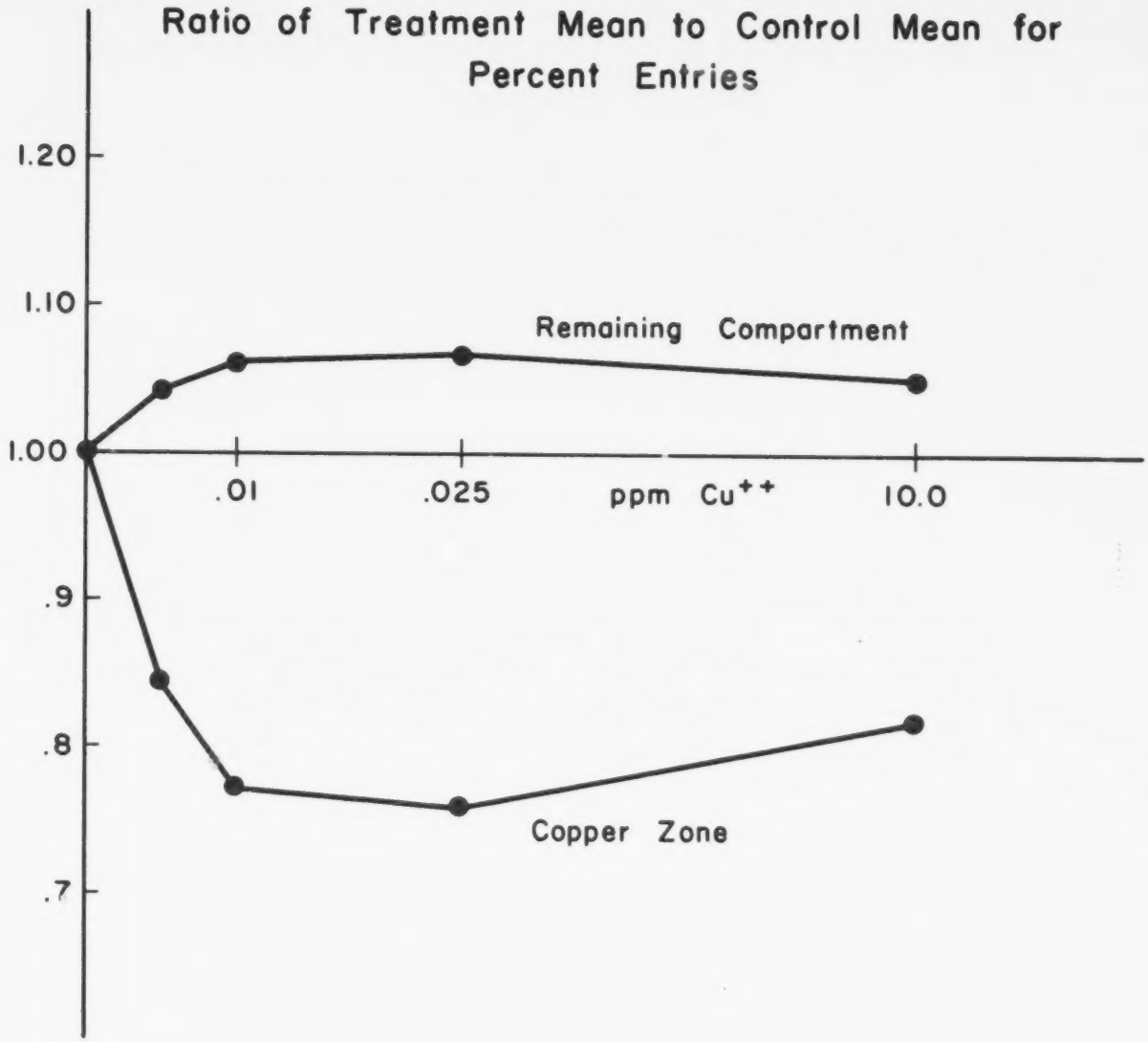
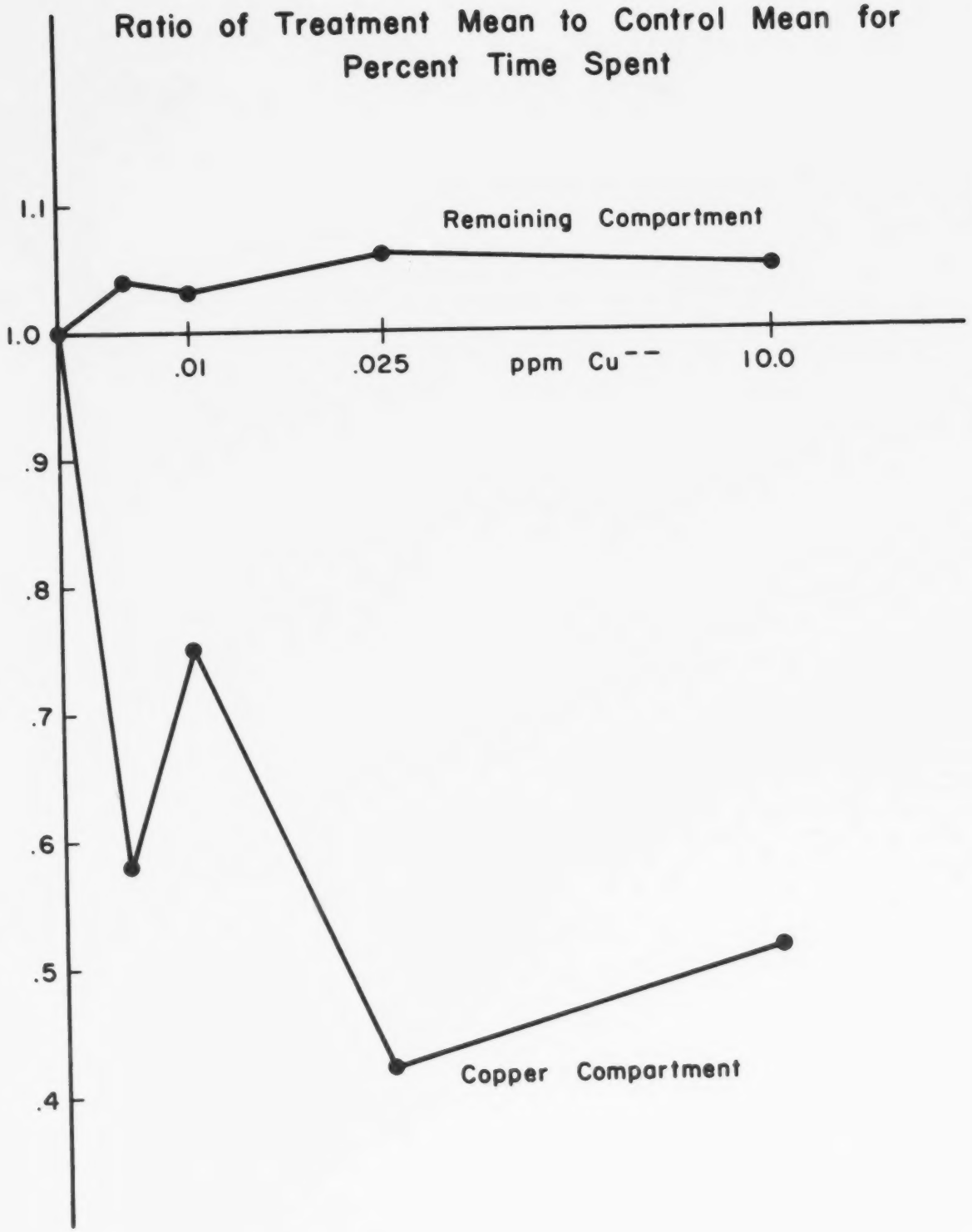

Ratio of Treatment Mean to Control Mean for Percent Entries
1.20
1.10
1.00
.9
.8
.7
Remaining Compartment
.01
.025
ppm Cu++
10.0
Copper Zone

Figure 13

The Ratio of the Treatment Mean to the Control Mean for Percent Time Spent in Compartments With and Without Copper (Monitor #2)


Ratio of Treatment Mean to Control Mean for Percent Time Spent
1.1
1.0
.9
.8
.7
.6
.5
.4
Remaining Compartment
.01
.025
ppm Cu--
10.0
Copper Compartment

SECTION XI

INTERACTION OF TEMPERATURE AND COPPER IONS AS ORIENTING STIMULI IN THE LOCOMOTOR BEHAVIOR OF THE GOLDFISH

The previous work on the orientation response of goldfish to copper ions distributed within discrete zones in a mass of copper free water demonstrated that the same copper concentration may elicit either "attraction" or "avoidance" behavior. The response exhibited seems to depend on the steepness of the gradient leading from the mass of copper free water to that containing the maximum copper concentration.

Clearly, the interaction between the copper ion proper and the gradient creates stimulus conditions which cannot be accounted for by the concentration of copper ions alone. The aim of the following experiments was to ascertain whether a similar interaction occurs between temperature and copper in their combined effect on the orientation of locomotion in the goldfish.

By means of an infusion withdrawal pump, $CuCl_2$ stock solution was infused into the water supply leading to each of two of the 16 compartments of monitor #2 so as to produce there a copper concentration of 0.01 ppm Cu^{++}, with negligible variation due to the above-mentioned fluctuation in flow rate of the water supply. All copper concentrations were checked by the atomic absorption technique.

To produce a temperature differential between compartments of 0.4C, the maximum which could be obtained without raising the temperature of neighboring compartments, heating pads were used. Each pad shaped to the contour of the compartment, was a nearly flat, black, silicone rubber mat in which resistance wire was embedded.

Copper infusions and heating pads were arranged so as to obtain the conditions illustrated in Table 10 and figure 14.

In all experiments the relative orientation of the five conditions was the same, but before each experiment their absolute position in the tank was changed randomly.

A single fish was introduced into the tank after the desired copper concentration and temperature increase (0.4C) had been attained in the selected compartments. In each of the 16 experiments, done during July and August 1972, the movements of a single fish were monitored during 24 hours following its release in the tank.

The 16 goldfish (25-33 cm) were obtained from a commercial grower in central Texas and kept during several months in large holding

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Table 10. Designation of the five experimental conditions in the monitor tank.

Condition	Number of Compartments Affected	Heating pad functional + non functional- not present 0	Cu^{++} present + not present -
A	one	-	+
B	one	+	-
C	one	+	+
D	one	-	-
E	twelve	0	-

tanks supplied with recirculating, carbon-filtered water which was 1-1.4 C cooler than the water used for the experiments.

A plastic bag containing a fish in water from its holding tank was suspended for 25-30 minutes in the water of the monitor tank prior to the release of the animal to allow gradual adjustment to temperature differences.

The percent distributions of the frequency of entry and time spent in each compartment were obtained for each experiment. All these distributions were rotated so as to line up the four compartments representing conditions A to D, which were then considered to be the same in subsequent analyses. A two-way analysis of variance for frequency and time spent was used to test whether there were significant differences between compartments, and a condition x fish interaction. Variability between fish was eliminated by blocking and bias due to intangible physical factors was minimized through a randomized assignment of experimental conditions to the compartments. The variability due to conditions was partitioned into contrasts and selected treatment comparisons to isolate the effects of each condition. In addition, the means for time spent and entries were plotted to obtain the effect of copper and of heat. (Figures 15 to 18).

Results and Discussion: The effects of the copper ion and of the temperature differential, illustrated separately in figures 15 to 18 are based on an analysis of variance.

Whereas the presence of the copper ion in the absence of heat (condition A) brings about a significant decrease in both percentage of entries and time spent per entry, temperature rise in the presence of copper (condition C) produced a significant increase in both the percentage of entries into compartments and the time spent per entry.

Although temperature rise in the absence of the copper ion leads to a significant increase in both percent entries and time spent per entry, the same temperature rise in the presence of copper further increases the percentage of both variables significantly. Figures 15 to 18 clearly demonstrate that the described effects are distinct from those produced by the presence of the heating per se.

It is concluded from the above evidence that in the experimental conditions described, the fish display a trend to "avoid" the copper-containing compartments by a relative decrease in both the number of entries and in the time spent in a compartment per entry. However, when the presence of copper is associated with a 0.4 C rise in temperature, "avoidance" changes to "attraction", with significant increase in the percentage number of entries as well as in the time spent per entry in the copper and heat compartment.

Figure 14

Arrangement of Experimental Conditions of Temperature and Copper in Monitor #2

Figure 14. Arrangement of experimental conditions in the monitor tank.

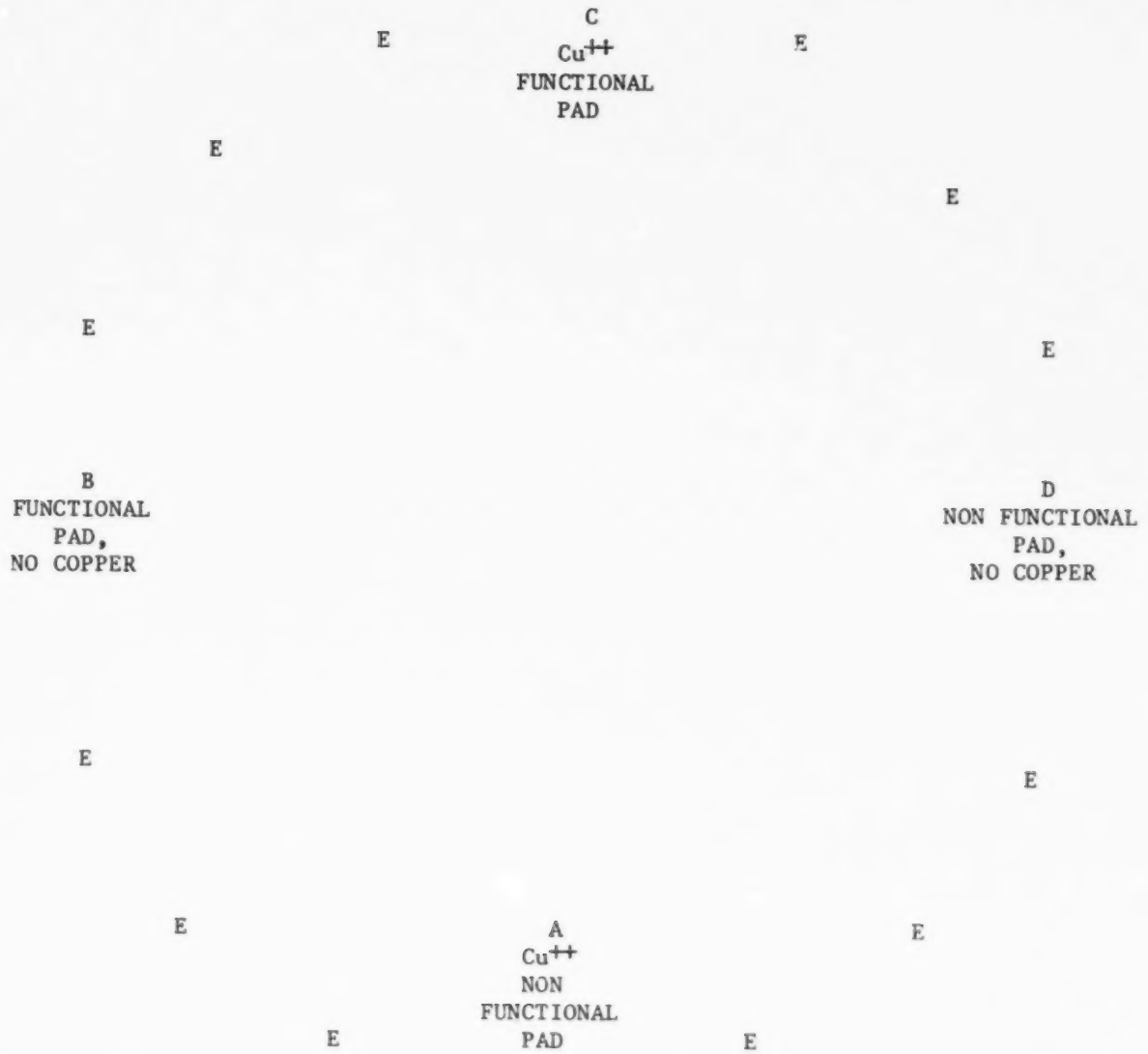


Figure 15

Effect of Heat on the Mean Percent Time Spent in Compartments With and Without Copper (Monitor #2)

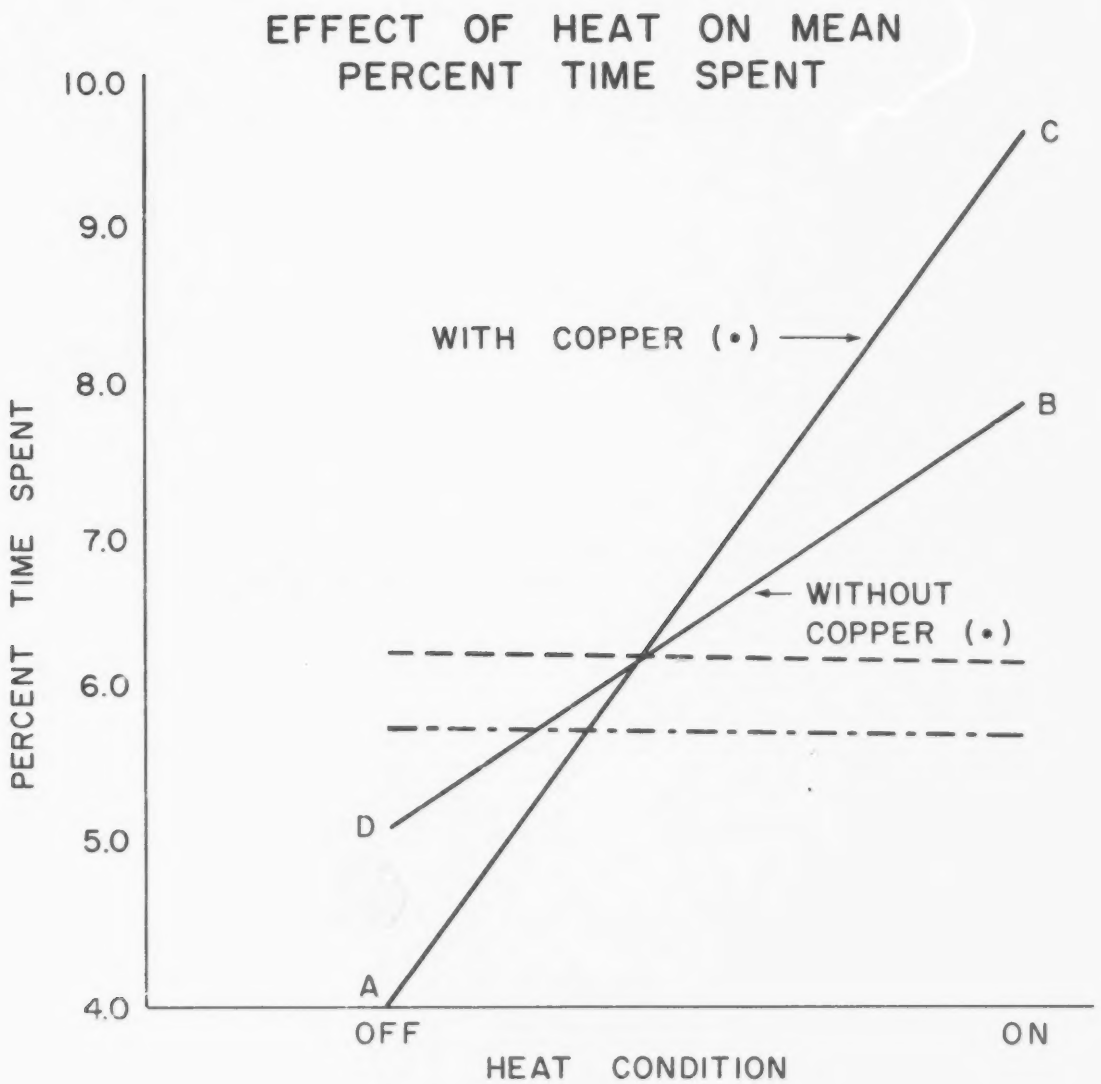

EFFECT OF HEAT ON MEAN
PERCENT TIME SPENT
10.0
9.0
8.0
7.0
6.0
5.0
4.0
PERCENT TIME SPENT
C
WITH COPPER (•)
B
WITHOUT
COPPER (•)
D
A
OFF
ON
HEAT CONDITION

Figure 16

Effect of Heat on the Mean Percent of Entries into Compartments With and Without Copper (Monitor #2)

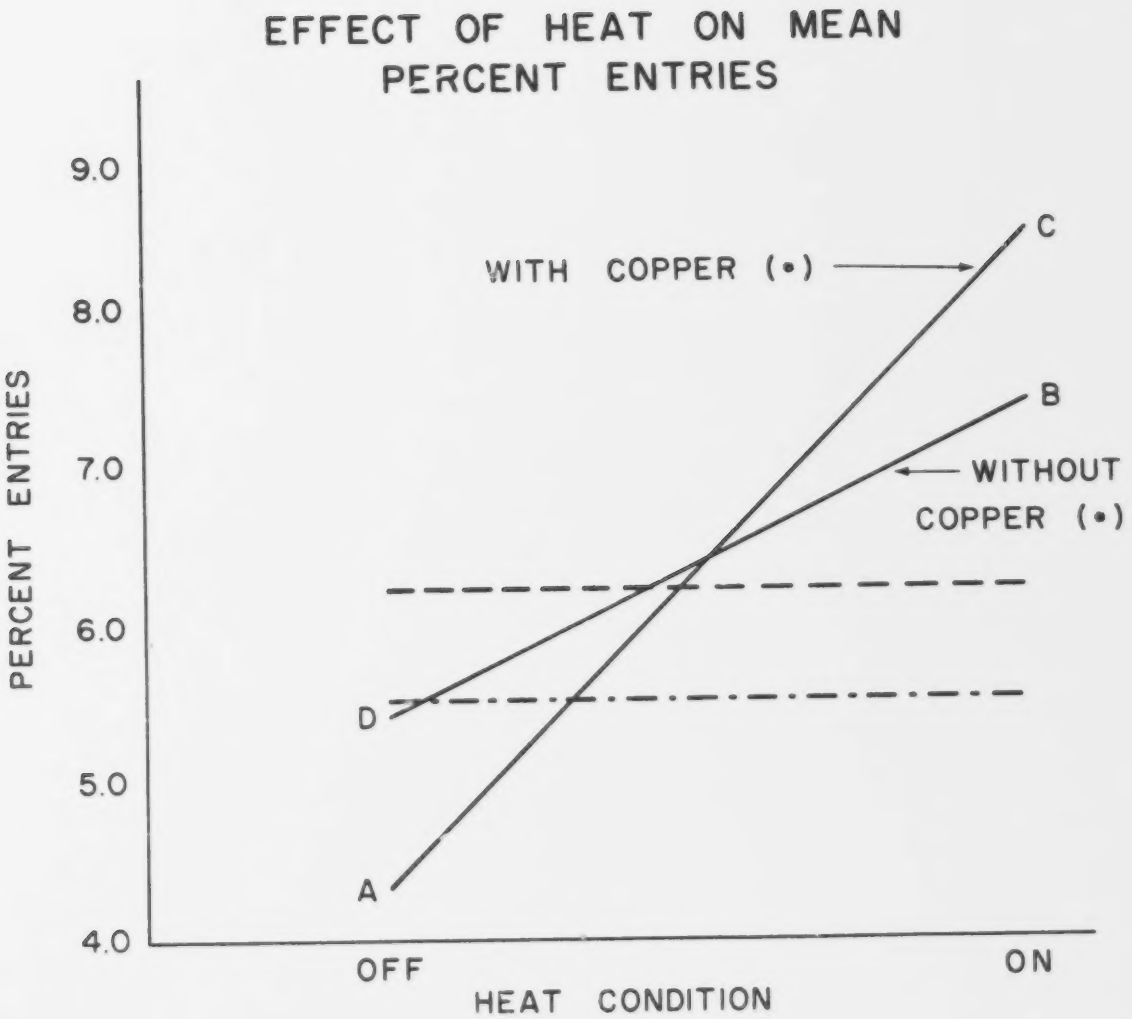

EFFECT OF HEAT ON MEAN
PERCENT ENTRIES
9.0
8.0
7.0
6.0
5.0
4.0
PERCENT ENTRIES
WITH COPPER (•)
WITHOUT
COPPER (•)
A
B
C
D
OFF
ON
HEAT CONDITION

Figure 17

Effect of Copper on the Mean Percent Time Spent in Compartments With and Without Heat (Monitor #2)

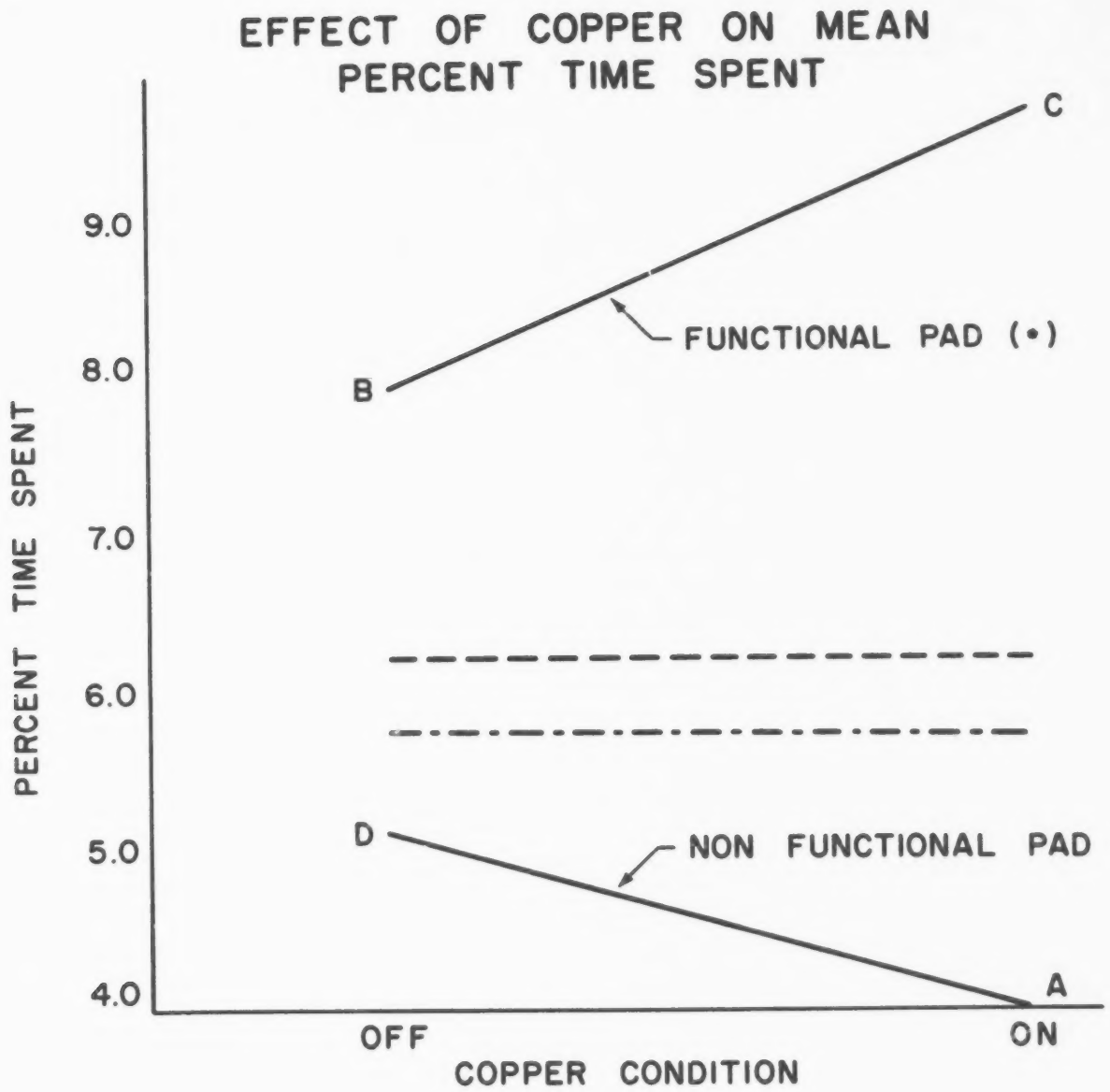
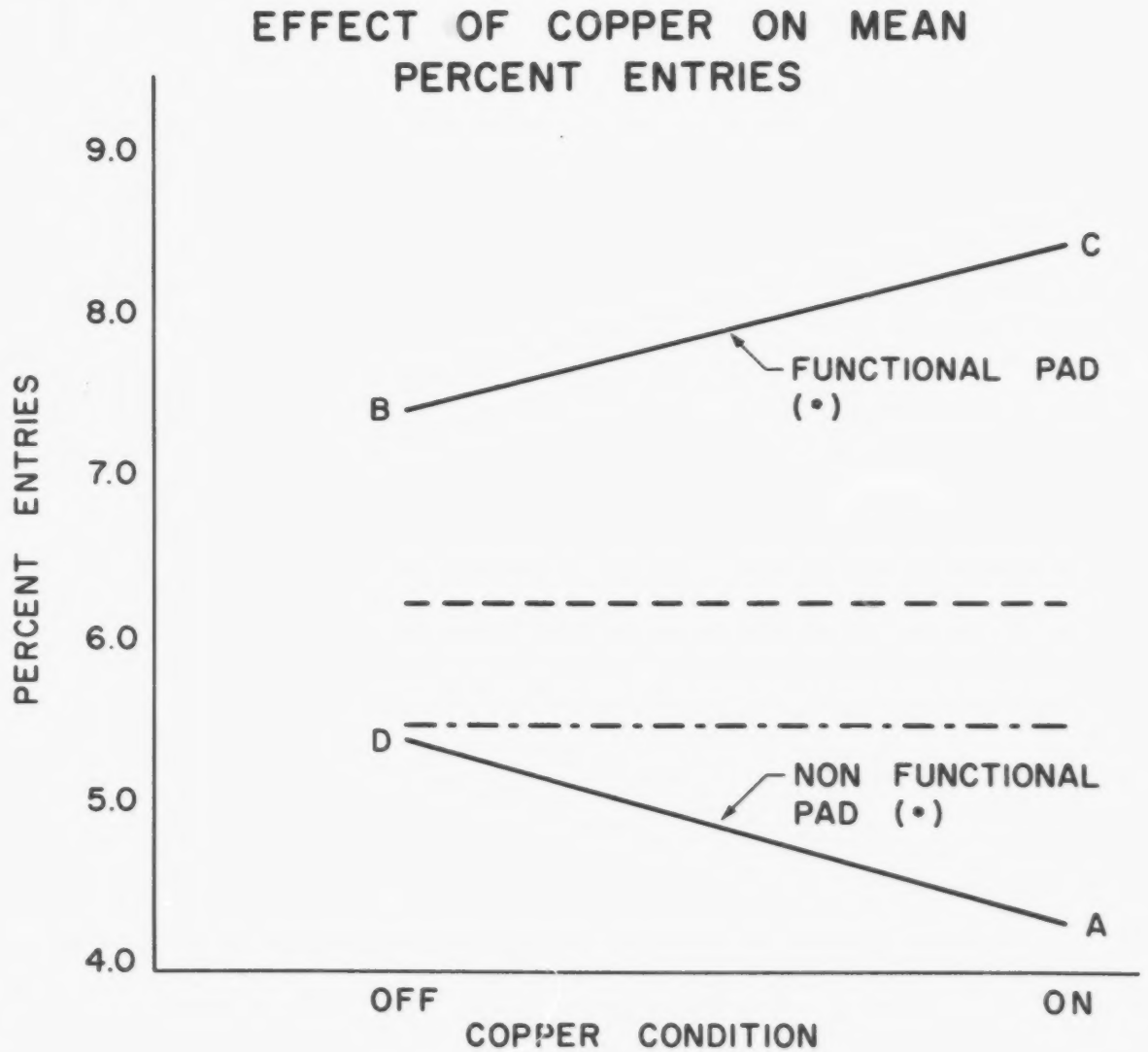

EFFECT OF COPPER ON MEAN
PERCENT TIME SPENT
PERCENT TIME SPENT
9.0
8.0
7.0
6.0
5.0
4.0
C
B
FUNCTIONAL PAD (•)
D
NON FUNCTIONAL PAD
A
OFF
ON
COPPER CONDITION

Figure 18

Effect of Copper on the Mean Percent Entries into Compartments With and Without Heat (Monitor #2)


EFFECT OF COPPER ON MEAN PERCENT ENTRIES
PERCENT ENTRIES
9.0
8.0
7.0
6.0
5.0
4.0
B
C
FUNCTIONAL PAD (•)
D
NON FUNCTIONAL PAD (•)
A
OFF
ON
COPPER CONDITION

Although there is an extensive literature on the effects on locomotor orientation in fish of either temperature or heavy metal ions individually, no reference could be found to interaction between temperature and these ions, such as that described here.

SECTION XII

THE LOCOMOTOR RESPONSE TO COPPER IONS IN THE WHITE SUCKER CATOSTOMUS COMMERSONI COMMERSONI) AND GREEN SUNFISH (LEPOMIS CYANELLUS)

White Sucker

The experiments done on the goldfish, channel catfish and largemouth bass were repeated on the white sucker (14 fish) in monitor #1.

The basic locomotor behavior of this species is sufficiently different from the others to require a different statistical treatment of the data. The fish display a high degree of thigmotaxis which results in the locomotor pathway being almost exclusively along the walls of the monitor tank. In the virtual absence of locomotion in the remainder of the tank the usual statistical treatment by which the locomotion in equal areas of the monitor is compared had to be modified. Analysis of variance and treatment contrasts for the control, NaCl, and $CuCl_2$ conditions were applied. Each condition lasted eight hours.

Results: With exception of length and frequency of straight pathways (or steps), the presence of the copper ion did not significantly affect the locomotor characteristics of the white sucker. The length of the straight pathways became significantly shorter in the copper region while their frequency increased highly significantly.

The data indicate that the time spent by the fish in the copper region increased. However, the peculiar locomotor behavior of this species makes the interpretation of the data analysis more difficult than with the other species studied.

Green Sunfish

Twelve fish of this species were tested in monitor #1 using the experimental method and data treatment described in Section X at a concentration of .050 ppm as $CuCl_2$. The fish spent more time in the copper-containing region than in the "non-polluted" region of the tank. Three locomotor characteristics were affected by the presence of the copper: the frequency of turning and of straight paths increased.

Introduction of the copper ion resulted in a significant increase in the time spent by the fish in the copper-affected region while the frequency of turning also increased. The remainder of the locomotor characteristics analyzed was not affected with exception of the total activity which consistently increased during the eight hour intervals of copper chloride infusion.

SECTION XIII

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Present addresses:

* Center for Environmental Studies
Virginia Polytechnic Institute and
State University
Blacksburg, Virginia 24061

** Department of Zoology
University of Western Ontario
London 72, Ontario, Canada

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